

Death of a secular saint

Moscow's mourning of the death of Sakharov is understandable, but Sakharov's life has touched all of us in ways that the international research community dares not forget.

IN other times and places, Andrei Dmitrievich Sakharov, who died last week, would have been made a saint. For the past quarter of a century in the Soviet Union, he lived as one. Since his return from exile in Gorky in 1986, the high and mighty have trudged to the tiny kitchen of his Moscow apartment, seeking enlightenment and a little courage. Like the classical saints, Sakharov taught by example, often breathtaking example.

Although all of us everywhere are now in Sakharov's debt, Mr Mikhail Gorbachev is the most conspicuous beneficiary of his turbulent career. The scandalous decision to exile Sakharov was a bankrupt administration's attempt to silence the most distinguished critic of its way of doing business with the courage to speak his mind within the Soviet Union. Exile would have served its purpose if Sakharov had been more easily cowed. In the event, his fortitude during these six years, and his puckish ingenuity in arranging that his opinions continued to circulate, eventually helped to force the election of a reformer as general-secretary of the Soviet Communist Party and then made possible the goal of *perestroika* on which Gorbachev has set his sights. Although Sakharov, pushing for faster change, has latterly been a thorn in his leader's side, Gorbachev did well to acknowledge his debt on Monday morning.

Sakharov's wider contribution to the new temper of Soviet life is the example of his disarming and, often, infuriating directness. His habit was to say what he believed, about personal liberty, nuclear weapons or even the dilemma of Nagorno Karabakh, without calculating in advance what his listeners would think of him for his opinions. Much of the turbulent Congress of People's Deputies now in session has taken its cue from Sakharov. The benefit is that the Soviet people now know how desperate is their plight.

The rest of us have a more subtle debt to pay, arising from Sakharov's sense of professional rectitude. Sakharov was conscripted into military research during the Second World War and, by 1945, had become one of the small group of theoreticians canvassing the feasibility of both the peaceful and the military uses of thermonuclear fusion. As the world

knows, the military project was carried through successfully, and Sakharov rewarded with the Order of Lenin (snatched away again when he was exiled). But, from the outset, Sakharov insisted on the dangers inherent in this enterprise. During his own exile to Gorky, he pleaded publicly the cases of others whose plight was even worse, urging that the international community of scientists, "the one real worldwide community that exists today", should shoulder the responsibilities its privileges imply (*Nature* **291**, 184; 1981). How well will the responsibility be discharged now that the prophet is dead? □

Leaders lead, OK?

With Sakharov gone, Mr Mikhail Gorbachev will have to change his style.

How should Mr Gorbachev comport himself in the interval between now and the time when there is another gadfly to turn attention from his own subversiveness? He himself must fill the vacuum left by Sakharov. But how?

On one cogent view, *perestroika* has already run into the ground: there is nothing in the shops. At a more abstract level, the rouble is overvalued, meaning that there will have to be a real (not a fake) devaluation in the next few months. People will have to pay more for luxuries, of which they have precious few as things are. Then there may be room for a true price reform, from which even Gorbachev has so far shrunk for fear of the trouble it will cause. Between now and a year from now, Gorbachev has to resolve the economic crisis. So far, luck has been on his side.

The truth, much more encouraging, is that time is also on Gorbachev's side, if only in a narrow sense. So much is apparent from the past few weeks, but here is how that logic goes. Who would have thought, just a few weeks back, that the Soviet enterprise required a new regime in East Germany? Now there is such a one, and also in Czechoslovakia. At this rate, it will be Romania next, or even Albania. For the time being, Gorbachev cannot but be pleased; people, it appears, are on the side of change. But how quickly will change be allowed to happen? Not particularly quickly, it appears. In the short run, Gorbachev will have to face the greater difficulty of saying where he stands. Without Sakharov, that will not be easy. □

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LIBRARIANS please note: A production problem caused a discontinuity between the end of the 30 November issue (page 578) and the start of the 7 December issue (page 599). The 'missing' pages, 579–598, have not been allotted.



Moscow bids farewell to the nation's dead hero

- Academician Andrei Sakharov is buried
- A city pays its respects

Moscow, Monday

THE nation's grief has never been so all-pervading and sincere as now, when Moscow bids farewell to Academician Andrei Sakharov, the "conscience of the nation". The sense of loss compares in its magnitude only to that at Yuri Gagarin's funeral.

The days that have passed since Sakharov's death on 14 December have shown graphically what profound respect he commanded among the people — this once-defamed man of harsh destiny, the father of the Soviet H-bomb, who spent years in exile under Brezhnev for advocating human rights; the man who bewildered the world with dazzling intellectual revelations and brought it the ideas of new thinking, new relations between different systems; and, finally, the first Soviet holder of the Nobel peace prize.

On 17 December, the temperature in Moscow fell to 20 degrees below zero Centigrade. People fell into long and silent lines along Komsomolsky Avenue where the coffin with Sakharov's body was placed in the Youth Centre. The traditional guard of honour over the body was absent this time: instead, taking shifts every 4 or 5 minutes, were People's Deputies, scholars, Sakharov's colleagues in research and public life, members of the Academy of Sciences of the USSR, poets, writers, representatives of numerous democratic movements and societies.

The ceremony ended at midnight instead of the scheduled 5 p.m. A simple yet moving token of the nation's tribute to the dead academician was a heap of red carnations piled up near the coffin, adorned with funeral wreaths contributed by official and unofficial organizations from all parts of the country.

Today, the ceremony has continued at FIAN, the Lebedev Physical Institute, whence a huge manifestation column set out behind the coffin with Sakharov's body, carried on people's shoulders to the Luzhniki Stadium, where the ceremony became a truly national memorial meeting.

The image of the contentious academician will persist in people's memory. A brilliant physicist-theoretician, he became an academician at 32, the youngest in the Soviet Union. His work on the hydrogen bomb, pioneer ideas on thermo-nuclear synthesis and a number of discoveries in other fields brought him recognition as an outstanding scientist.

He was decorated with the Lenin Order, three Stars of the Hero of Socialist

Labour, and given the Stalin Prize — and prohibited from leaving the Soviet Union for reasons of state secrecy.

"Among our scientists, he was, as the saying goes, a transparently-honest man", wrote Nikita Khrushchev in his memoirs. "He hated to think that science might be used for the destruction of life, poisoning the atmosphere and slow killing of the people by nuclear pollution."

Yet it was Khrushchev who failed to

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Paying tribute — a long line of Muscovites queue outside the Youth Centre.

heed Sakharov's request not to resume nuclear tests in 1961. This is how Sakharov characterized the ensuing series of H-bomb test explosions: "It was terrible. I became a different man after that." He did not quit science, but concentrated on politics and free thinking, officially called dissent and equated to a crime against the state or a psychiatric disease.

It was hard to send Sakharov to a psychiatric hospital and he thus became a symbol of conscience and hope. The desperate letters imploring him for help often bore a simple address: "Academician Sakharov, Moscow". And he undertook to defend the persecuted in the press, in court rooms and in public, even when he realized that his efforts would be in vain.

The Brezhnev system took up the challenge. Without official accusation and trial, Sakharov was sent to the city of Gorky, inaccessible to foreigners for state security reasons. (The exile ended in 1986 with a telephone call from Mikhail Gorbachev.) Academicians denounced him, but he was never expelled from the academy even in the darkest days of witch-hunting, much to the credit of that

institution. He stayed in the Soviet Union, having retained his firm civic stand, unperturbed by any persecutions.

In his book *Reflections on Progress, Peaceful Coexistence and Intellectual Freedom*, published in the West in 1968, he called for the elimination of the arms race and the solution of global issues — hunger, racism, disproportionate development of nations and environmental pollution. In 1975 he wrote a book *On Knowledge and the World*. The ideas he developed in that book are obviously parallel to those advanced and discussed in the Soviet Union now.

People's Deputy Sakharov considered *perestroika* to be an event of major magnitude for the Soviet Union and the entire world. He wanted this process to be more speedy and radical in this country. He perceived *perestroika* as a harbinger of the times when the patient Soviet people's long-cherished aspirations and hopes would finally come true.

By his nature, he could not stay aloof from what was going on in his country and abroad, and spoke at the Second Congress of People's Deputies on the day of his death. One of the leaders of the parliamentary inter-regional group, Sakharov was loyal to the end to his absolute and irreconcilable civic stand. The nation will forever remember him as a true patriot, a citizen of his country and the world, a person with an open and generous heart and a fighter for justice and the priority of common human values.

Academician Andrei Sakharov was buried at the Vostryakovo Cemetery in Moscow on 18 December 1989.

Yuri Kanin

Moscow correspondent
Novosti Press Agency

SPACE STATION

A Mir \$10 million

Tokyo

THE Soviet Union has some bargains going in slightly used space stations. But do not rush — the Japanese were there first. Last week, in one of the oddest sales of the decade, a tiny Japanese company capitalized at just \$7,000 bought the back-up Mir space station for \$10 million.

The Horie Group company has only ten employees and usually does nothing more exciting than organize seminars on space. It bought the station while it was on display at World Design Expo 1989, held in Nagoya from 15 July to 26 November. The space station had been on display earlier at exhibitions in France and Canada but there were no takers.

Horie Group says it has no immediate plans to launch the space station but it will allow Japanese companies interested in developing space technology to study it.

David Swinbanks

CANCER DRUGS

Restrictions recommended

Tokyo

AN advisory body to Japan's Ministry of Health and Welfare is expected to recommend restrictions this week on the use of the world's two top-selling anti-cancer drugs, Krestin and Picibanil.

The drugs are sold only in Japan and bring in hundreds of millions of dollars each year. But they have become the centre of controversy because of their questionable efficacy.

The Central Pharmaceutical Affairs Council is expected to rule that from now on the two drugs should be prescribed only in combination with other chemotherapeutic drugs and should not be prescribed alone. But a leading critic says the council's decision is unlikely to have much effect on prescription of the drugs and claims that there are serious defects in the process of drug re-evaluation which throw into question the council's impartiality.

Both drugs are so-called biological response modifiers — agents that change the body's response to tumour cells. Krestin is an extract of fungus and Picibanil is a penicillin-treated freeze-dried streptococcus. Both were put on the market in the mid-1970s and their combined sales rose rapidly to about ¥80,000 million (\$570 million) a year, which is an order of magnitude larger than sales of anti-cancer drugs in the United States, for example (see Commentary, page 850). Total sales so far amount to more than ¥1 million

ENVIRONMENT

Japan accepts ban on drift-net fishing

Tokyo

UNDER international pressure, Japan last week agreed to stop using massive fishing nets to catch tuna and squid in the South Pacific Ocean, at least for the time being.

Japan, Taiwan and Korea have been severely criticized by South Pacific island nations, New Zealand, the United States and environmentalists for using drift nets up to 60 km in length in the Pacific Ocean (*Nature* 342, 9; 1989).

Last month, Japan and Taiwan refused to join an international ban on drift-net fishing in the Pacific (see *Nature* 342, 726; 1989). But Japan made a quick about-turn last week to avoid conflict with the United States.

The UN resolution endorsed by Japan last Friday calls for a ban from 1 July 1991 in the South Pacific and worldwide from 30 June 1992. But the ban can be waived if "effective conservation and management measures" are taken. And some environmentalists fear that this may provide Japan with a loophole. David Swinbanks

million (\$7,000 million).

Cancer experts have questioned the efficacy of Krestin and Picibanil for years and in 1987 the Ministry of Health and Welfare decided to re-evaluate them. Details of the council's recommendations had not been officially released when *Nature* went to press. But a ministry official confirms that recent reports in the Japanese press of the restrictions to be imposed are "more or less accurate".

Masanori Fukushima of Aichi Cancer Center, who has been campaigning to reform Japan's drug approval system, says the reported decision to allow these drugs to continue to be prescribed in combination with other chemotherapeutic agents represents a "gigantic loophole". The drugs are already usually prescribed in combination with other anti-cancer agents. And a spokesman for Kureha Industrial Company, which developed Krestin, has confirmed that Krestin is "nearly always" prescribed with other chemotherapeutic drugs.

Fukushima questions the ability of the the council's subcommittee on anti-cancer drugs to re-evaluate Krestin and Picibanil impartially. Fukushima points out that two of the seven subcommittee members carried out some of the clinical trials on Krestin which the subcommittee had to re-assess. And a third member is on the editorial board of the journal *Oncologia*, which published their results. Furthermore, *Oncologia* was established and is financed by Kureha.

A Ministry of Health and Welfare official says that the overlap between members of the subcommittee and those that carried out clinical trials "cannot be helped" because the number of suitable experts in Japan is limited. He says that the root of the problem is the lack of government finance for medical research. He points out that the ministry, which finances the larger part of Japan's medical research, has a budget of only about ¥50,000 million (\$350 million) per year, less than one tenth that of the US National Institutes of Health. And so "inevitably" Japanese medical researchers have to seek the financial support of pharmaceutical companies by carrying out clinical trials.

He says that almost all Japanese pharmaceutical companies have their own in-house journals for quick publication of clinical trial results. And while sympathizing with Fukushima's proposal that all clinical trial results used in drug assessment should be published in English in international journals he says this is impractical because of the time it takes.

But Fukushima says that there are plenty of internationally known Japanese researchers capable of carrying out assessment of clinical trials. David Swinbanks

UK UNIVERSITIES

Selling student places

London

THE heads of UK universities last week received details of a new system of competitive bidding for undergraduate places in a letter from the Universities Funding Council (UFC). By June 1990, each university will have to say how many undergraduate places it wishes to make available in each subject area by the 1994–95 academic year, and make an 'offer price' per student for each subject.

Guide prices will be issued within the next few weeks, but the letter notes that "in some (if not all) subjects, universities may wish to... improve their chances of having their offers accepted in full by quoting a price below the guide price". Where lower offer prices are made, UFC will leave it to individual universities to show that "adequate arrangements exist for assessing... academic standards".

After assessing the bids, UFC will inform each university by early 1991 of the number of student places to be supported and the prices for teaching that have been accepted for the 1991–92 academic year, together with provisional figures for the three following years. As well as cost and teaching quality, the UFC says the range of subject provision and the balance between different subjects will be taken into account in the allocation of student places and teaching grants to the universities.

Some subject areas are excluded from the competitive bidding system. Student numbers for medicine, dentistry and veterinary science will be allocated to universities by the UFC and supported at the relevant guide price.

The new system reflects the government's desire for a more market-orientated university system. Sir Peter Swinnerton-Dyer, chief executive of UFC, says that the new arrangements will "increase institutional autonomy and widen student choice as the balance of public funding for higher education moves from centrally provided grants to fees".

The government expects student numbers to increase by just under 10 per cent between 1988–89 and 1992–93, and for there to be a shift towards science and technology. UFC expects an increase of at least 10 per cent by 1994–95 but the exact figure will depend on the universities' bids.

The Committee of Vice-Chancellors and Principals (CVCP) was concerned at its meeting last Friday that funding for the universities for 1990–91 may not be sufficient to provide "satisfactory pay settlements". It has also announced the formation of an 'Academic Audit Unit' to review each university's mechanisms for monitoring academic standards. A full statement on the competitive bidding system is now expected in mid-January, after the next CVCP meeting.

Peter Aldhous

DNA fingerprinting on trial

Washington

A DRAMATIC series of events in a Portland, Maine, courtroom seems certain to lend strength to those calling for stricter standards in the forensic use of DNA testing.

In a preliminary hearing to determine whether evidence from DNA-typing is "sufficiently reliable to be held relevant" as evidence, the prosecution abandoned its case after a series of blunders were revealed under cross-examination.

The prosecution withdrew its DNA-print evidence without the defence having to present a single witness.

The prosecutor, Deputy District Attorney Laurence Gardner, says he has complete faith in the value of genetic fingerprinting but criticized Lifecodes Corporation, the company that prepared the DNA evidence for him, for failing to inform him "of any problem areas that were going to come up".

The trial is the first upset for Lifecodes after the celebrated case in which DNA fingerprint evidence was found unreliable in a 12-week hearing in a Bronx court (see *Nature* 339, 501; 15 June 1989). The company has successfully presented DNA evidence in some 80 other cases and claims that it is "happy with the results generated" in the Portland case.

Lifecodes spokeswoman Karen Wexler says that the problems in Portland were "in our opinion, more of a function of the prosecutor's willingness to go on with this case, in terms of a commitment of time and perhaps finances to get expert witnesses".

The case involved the sexual molestation of a five-year-old girl. At issue was whether a DNA fingerprint prepared from a semen sample found on a tissue at the scene of the crime matched that prepared from a blood sample taken from the defendant. The 'fingerprint' is the pattern of bands produced when specific fragments of DNA that vary in size according to an individual's genetic makeup are separated out on a gel.

The issue that eventually divided the court was the method Lifecodes used to decide that the patterns on the two gels matched. A straightforward match was not possible because the two fingerprints could not be simply superimposed — all the bands on one gel had run faster than in the other.

Such 'band shifting' is no surprise, as the speed with which DNA migrates across the gel depends on many factors — degree of degradation, contaminants and so on. But Lifecodes' way of correcting for the bandshift had not been challenged in court before.

The method is simple. A non-polymorphic marker (a marker for a DNA frag-

ment that is the same in all people) is used as a control to estimate the size of the band shift between the two samples. The same correction factor is then applied to other bands on the gel. In this case, a correction calculated by using the non-polymorphic marker DXZ1 generated a match between the two gels. "That indicates", said Gardner, "you could say the evidence and the defendant's blood had a common origin. That was my case".

But then came the bombshell. The defence had in its possession a piece of paper that appeared to show that Lifecodes had also tested a second non-polymorphic marker, DYZ1. But that probe seemed to generate a completely different correction factor, one that would mean the two samples would not match. Astonishingly, the paper appeared to have been sent to the defence by mistake among other papers they requested.

On the witness stand, Michael Baird of Lifecodes agreed that it should be possible to substitute DYZ1 and DXZ1 "to correct for the migrational difference between the DNA in the evidentiary and the [defendant's] sample". But Baird was then presented with the piece of paper, in his own handwriting, which gave a completely different correction factor using the DYZ1 marker.

"I was devastated. The court was devastated", said Gardner. He had found out that a different non-polymorphic probe gave a different result, "because the defense attorney has a summary fragment sheet in Michael Baird's handwriting that I have never seen. That they never showed to me. That they sent to the defense by mistake." And it appeared from what had happened that Lifecodes had done the most unscientific thing imaginable, which was they had hidden data, not disclosed data that did not agree with their conclusion.

That is not actually true, as Gardner now knows. The reality is that the situation is more complicated than Lifecodes' original explanation suggested. A single non-polymorphic probe can be expected to document only band shifts for DNA fragments of a particular size. Other non-polymorphic probes may document different shifts in different regions of the gel. There may thus be no contradiction when different results are obtained with different non-polymorphic probes.

Gardner says that, "If they had told me up front that here is a problem . . . that you don't really apply the mobility shift across the gel, I could have blunted all that testimony, given the opportunity". But instead, Gardner was stuck with a situation where it appeared that Lifecodes had changed its testimony on the stand, at one moment apparently stating that one non-

polymorphic marker alone could be used to correct for band shifting and a little later that different corrections applied in different regions.

Gardner decided not to continue the case. Had he gone on, he would still have faced several more problems — the first being the need for Lifecodes to justify its new method. Although Lifecodes' method is clearly rational — if enough markers are used — experts say they know of no published work that deals with the issue. Lifecodes' reply is that they have data to show their correction method works, although the work has not been published and was not presented to the court.

Naturally enough, this response does not satisfy outside experts who argue that forensic methods need to be generally accepted as valid by the scientific community. But Wexler says that the fact that "it has not been published in a peer review journal does not necessarily mean it is not valid. . . . If the prosecutor were to put several expert witnesses on the stand, the FBI perhaps and some other people, all would agree this is a valid way to document the shift."

A further hurdle would still remain. Defence attorney Gene Libby argues that "even if you accept the change in testimony as being correct, they had not done the necessary experiments in this case". He says that they did not have enough non-polymorphic probes to cover the larger DNA fragments and could not have compensated correctly throughout the size range of the gel. Whether his argument is correct is now academic as the Portland case is very unlikely to be reopened.

Defence attorney Gene Libby is clearly pleased by his victory. He had spent a month "studying molecular biology and genetics" before the case and says his example will show other defence lawyers that "this type of evidence can be challenged successfully and it is not such hard science that it is a waste of time to seek to challenge it".

Alun Anderson

■ THE prime source of guidance on the use of genetic fingerprinting now seems likely to be the National Academy of Sciences. The academy announced last week that it had finally won the finances and approval to carry out a 14-month study on DNA Technology in Forensic Science.

Victor McKusick of Johns Hopkins University will chair the 14-person panel. Other members will be named shortly and a first meeting is planned for January.

The project has been planned for some time but financial support has been slow in coming. About \$250,000 has now been found from the Federal Bureau of Investigation, the National Institute of Justice, the National Science Foundation, the National Institutes of Health and the Sloan Foundation. □

ANIMAL RIGHTS

German research fights back

Munich

THE naming of the first "animal protection official" in West Germany has mobilized some of the country's top researchers in defence of the use of animals in research. Although protests from researchers have already led to limits on the power of the new official, the creation of the post reflects the growing political power of animal rights activists. Important changes in the way research with animals is regulated and monitored by the state may follow.

Researchers were so confident of their position that they supported Minister-President Walter Wallmann (Christian Democrat) of the *Land* of Hesse when he created the new post. Researchers felt that their right to do experiments using animals was effectively protected by West German law.

Every experiment must be approved by a committee consisting of a two-thirds majority of 'experts' from the fields of medicine, biology and veterinary science.

But the man Wallmann nominated, the journalist and animal rights activist Ilya Weiss, was seen as the last person researchers wanted in the job. Researchers are now worried that the fashion of having an 'animal ombudsman' may spread to other *Länder* or to the federal government.

Weiss, who is 36 years old, is due to begin his duties on 1 January. He has been one of the most ardent advocates of the animal liberation movement in West Germany and publicly supported such actions as the 'freeing' of macaques from the research unit of INSERM in Lyons (see *Nature* 339, 407; 1989). Weiss was the head of a West German animal rights organization, and has conducted a 14-year crusade against the abuse of animals. He once locked himself in a huge cage in a central Frankfurt square for several days in order to dramatize the plight of animals used in research.

Weiss will have two full-time employees and an estimated budget of DM500,000 (\$290,000). His official task will be to observe the application of the West German Animal Protection Law in Hesse and to suggest improvements in the interest of animals in both agriculture and research. He will act as an agent for Hesse in animal protection matters on both a national and a European level.

On 30 November, Wallmann met with representatives of the employees' committee at Hoechst AG, the pharmaceutical manufacturer, which is based in Hesse. On 7 December, he met more than a dozen senior researchers and doctors, some of whom came from other *Länder* to warn him of the possible consequences of Weiss's nomination.

Wallmann assured both groups that

Hesse would remain an attractive location for research and industry and that he did not intend to limit animal experimentation "as long as it is necessary". Furthermore, he assured researchers that Weiss would have to clear all of his public statements with the government.

Researchers were disappointed, however, that Wallmann did not forbid Weiss access to the documents that researchers must file in order to gain permission to carry out experiments with animals. They fear that Weiss will pass on the contents of the files to animal-rights activists who might attack individual researchers.

Although animal-rights activity receded after the Animal Protection Act was redrafted by parliament in 1985, the potential for activity is still present — the national animal rights organization 'Deutscher Tierschutzbund' has 600,000 members, about one per cent of the population of West Germany. Peter Gutjahr-Löser, who follows the issue for the Max Planck Society, foresees an upsurge of activity.

A spokeswoman for the granting agency Deutsche Forschungsgemeinschaft (DFG), said that DFG preferred to wait and see what else happens in Hesse before taking any public action. It is important, she said, to see if Weiss does his job properly before responding.

Researchers are especially worried that they would lose support if they are forced to fight in public for the cause of animals

experimentation instead of just in private meetings with politicians. "If we had to go up against Weiss in a television debate, we would lose", said Wässle, because "we are amateurs and he is a trained professional." Weiss makes blanket statements condemning the use of animals, whereas researchers are trained to explain complicated issues in a qualified and cautious way, said Wässle. There is no doubt about which method comes across better.

Wässle rejected tactics such as sending leaflets to all the physicians in the country asking them to make their patients aware of the medical need for animal experiments. Although such tactics have been used by researchers in the United States, they would be seen as exploitation of the patients in West Germany, he said.

But not all the news is bad for researchers. The history of disagreements within the animal rights movement may reduce the threat to research. Some activists opposed the nomination of Weiss to the post in Hesse because they felt he was too radical.

Gutjahr-Löser admitted that Weiss's nominations "could have some positive effects" by raising the consciousness of researchers about animal rights. But researchers "do not need Weiss to make them think".

It would make more sense, he said, to hire more experts and veterinarians for the animal protection bureaucracy in order to speed up the application process, which has begun to fall far behind the timetable guaranteed by the law, and to increase vigilance against possible abuse of the animals.

Steven Dickman

ANTARCTIC RESEARCH

New Indian base planned on Weddell Sea

New Delhi

INDIA has despatched an expedition team on a chartered ship to the coast of the Weddell Sea in Antarctica with the eventual aim of establishing a new permanent base in that region. Known to be rich in mineral resources, it lies in an area where Britain, Chile and Argentina have overlapping territorial claims.

The 21-member expeditionary team, which left on 4 December, is led by Dr B.K. Raina of the Geological Survey of India. The team includes five scientists and 16 members of the Army, Navy, and Air Force. This is the ninth expedition to Antarctica since 1982, when the Department of Ocean Development (DOD) set up a station named Dakshin Gangotri at 70 degrees south and 12 degrees east. The station, located on shelf ice, began to drift and in 1986 the Indian team set up a second camp about 80 km inland. A permanent base has now been established there.

The expedition to the Weddell Sea area is a sign that India's Antarctic activities will

diversify. Vinod K. Gaur, the new Secretary of the Department of Ocean Development says the team will conduct geological studies, and that the department will eventually establish a permanent station there.

India is a consultative member of the Antarctic Treaty and has been a member of the Scientific Committee on Antarctic Research since 1984. So far, the Department of Ocean Development has spent about \$30 million on its eight expeditions.

The department intends to purchase a ship for future expeditions and has asked the government for about \$60 million for Antarctic research between now and 1995.

India does not recognize the territorial claims in Antarctica of any countries and says its present goals in Antarctica are purely scientific. But it has been stated officially that economic benefits for India can come from knowledge of the krill stock, and a fisheries vessel capable of operating up to 60 degrees latitude is being acquired.

K.S. Jayaraman

Talking of harmony

Washington

1989 ends with patent lawyers hopeful that at long last the holy grail of patenting — the international harmonization of patent laws — lies within their grasp, if not yet within their grasp. The reason for this surge of optimism is the unexpected success of the World Intellectual Property Organization (WIPO) set up last year in Geneva under UN sponsorship.

Unlike two earlier organizations that attempted harmonization of patent law, WIPO involves 121 countries, not just the patent offices of Europe, the United States and Japan. WIPO sponsored a successful meeting in Geneva in November at which patent experts examined the details of a draft harmonization treaty. It will sponsor a second meeting next June, with the aim of preparing a treaty for a diplomatic conference in June 1991.

The draft treaty that WIPO discussed in November would bring in some striking changes. The most contentious for the United States is a change from a 'first-to-invent' system to the 'first-to-file' system practised in Japan and Europe in which the patent is granted to whoever wins the race to the patent office. Although proponents of the US system contend that it is fairer, the procedures to determine who was first to invent are time-consuming and expensive.

Many of the clauses in the treaty are hotly disputed by all WIPO members, but one that meets with few obstacles is the plan for a uniform 'grace period' — the time before a patent application is filed during which the disclosure of a competing invention cannot influence the granting of a patent. The United States allows a grace period of one year and Japan six months but Europe has no grace period at all. This difference is one of the most troublesome for those filing for patents in several different countries. Most members agree on a 12-month grace period.

A rule that patent applications must be published no later than 18 months after they are filed is strongly supported, with objections only from the United States and New Zealand. The United States complains that it is unfair to publish a patent application because if the patent is not subsequently granted, valuable secrets may be given away. As the US patent office grants patents, on average, about 18 months after the date of application, the United States argues that there is no need for compulsory early publication, especially as much of the information is disseminated in scientific journals. But Japan argues that early publication benefits both everybody because it avoids a long period of insecurity during which the contents of patent applications remain unknown.

Japan's preference for early publication

is in part explained by the remarkable length of time it takes to examine patents. Not surprisingly, Japan objects to attempts to put a time limit of 18 months on examination of patents. Opposing the United States, United Kingdom, the Soviet Union and others supporting the limit, Japan argues that strict time limits have no place in an international treaty because many unpredictable factors, such as surges in the number of patent applications or a shortage of patent officers, could block even efforts made in good

EPO PATENT DISPUTE

Court battle ends at the start

Washington

ONE of biotechnology's longest-running battles was expected to end last week but now looks set to go into a new round of bargaining and bickering. On Monday 11 December a Boston court ruled that neither of the two protagonists — Genetics Institute of Boston and Amgen Corporation of Thousand Oaks, California — has a clear overall right to produce recombinant erythropoietin (EPO) by genetic engineering. In the short term, the ruling seems to favour Genetics Institute. EPO is a kidney cell protein that stimulates bone marrow red-blood-cell production, and annual worldwide sales are expected to reach between \$250 million and \$1,000 million.

The ruling, which is complex and runs to 184 pages, validates the central claims of the patents held by the two companies but at the same time doubles the confusion by considering both patents partially invalid and mutually infringing.

The Genetics Institute patent, issued in June 1987 after the company purified EPO from human urine, refers to "homogeneous human EPO", a term that the company claims to include recombinant EPO. The Amgen patent, issued in October 1987, covers the gene, vectors and cells required for production of recombinant EPO, although the patent did not specify the product itself.

Genetics Institute's version of recombinant EPO is called Marogen and is currently only in experimental use in the United States as it has not gained Food and Drug Administration (FDA) approval.

Amgen's recombinant EPO, called Epogen, gained FDA approval in June, 1989 and already has sales of more than \$50 million.

The most immediate effect of the court decision may be to make it easier for Genetics Institute to produce Marogen abroad. The Amgen patent has so far prevented Genetics Institute from manufacturing recombinant EPO in the United

faith to meet them.

One article in the draft treaty which could reduce the enormous number of patents currently clogging up the Japanese system gains universal support. The article broadens the meaning of 'unity of invention', so that technically related features of an invention are all protected under one patent, rather than under several different ones as is now common.

The question of patent lifetime provokes much argument. The draft WIPO treaty would provide 20 years protection. Uruguay is among those that argue that 20 years is too long, and India argues that the term should vary for different fields of

States. But it has been getting around the restriction by licensing Marogen to Chugai Pharmaceuticals of Japan and importing the product into the United States.

The new ruling found that offshore production of recombinant EPO by Chugai Pharmaceuticals does not infringe the Amgen patent, effectively putting an end to legal action by Amgen designed to prevent the import of Marogen. Even worse for Amgen, the ruling says that Amgen cannot produce Epogen in the United States without infringing the Genetics Institute patent. That puts Amgen in the same boat as Genetics Institute, which cannot produce recombinant EPO without infringing Amgen's patent. Amgen says that the ruling is unlikely to affect the availability of Epogen to patients. The stage looks set for a tussle over some form of cross-licensing agreement.

But Amgen may try to gain an advantage by waiting for the outcome of actions associated with a wider EPO dispute. Amgen has submitted two EPO process patents. If they are granted, Chugai Pharmaceuticals would be prevented from importing recombinant EPO into the United States.

Amgen is also benefiting from 'orphan drug' status for Epogen, granted by FDA for the treatment of anaemia in patients with chronic renal failure. This provides Amgen with a seven-year monopoly in the United States (see *Nature* 339, 493; 1989). Marogen, which is awaiting FDA approval for sale in the United States, could receive equivalent status if the FDA declares it to be a product distinct from Epogen. If not, an agreement may be negotiated whereby the orphan-drug status of Epogen is shared with Genetics Institute in return for Amgen not paying royalties in a cross-licensing agreement.

Both parties may, however, wish to see a speedy resolution to the dispute as the market for EPO is huge. **Diane Gershon**

SPACE SHUTTLE

Rescue mission prepared

Washington

THE mission of space shuttle Columbia, scheduled tentatively for launch on 8 January 1990, will be the first occasion on which the shuttle performs a task that was originally intended to have been one of its routine activities — the return to Earth of a satellite put into orbit on a previous shuttle flight. If all goes well, the crew of Columbia will manoeuvre the shuttle next to a cylindrical canister known as the Long Duration Exposure Facility (LDEF), pull it into the cargo bay, and bring it back down to Earth, where scientists will see the outcome of 66 experiments that have been sitting in the cold and the dark for five and a half years.

LDEF, put into orbit in April 1984, is a 12-sided cylinder, 30 feet long and 14 feet in diameter, holding 86 trays that contain a variety of simple experiments in physics, astronomy and biology. It was supposed to stay in low orbit for about a year, but before it could be retrieved, the Challenger explosion of January 1986 grounded the shuttle fleet for three years. This week's [planned] flight is an eleventh-hour mission: Columbia is the only shuttle with a cargo bay big enough to hold LDEF, and if the retrieval attempt fails, LDEF, now in a rapidly decaying orbit, will burn up in the Earth's atmosphere, probably by February next year.

The unexpected length of LDEF's flight, and the consequently aggravated effects of the upper atmosphere on its contents, could turn out to be the biggest problem for some of the experiments on board, according to Ernst Zinner, of Washington University in St Louis, Missouri, who is principal investigator of an experiment to capture interplanetary dust. Zinner's payload on LDEF consists of a number of 'sandwiches' of germanium plates in metallicized plastic foil coatings. Dust particles, travelling at speeds of 10 km per second, penetrate the foil but crash into the germanium and vaporize; the lower-speed fragments should then be retained by the foil coatings, so that the sandwiches retrieved from LDEF should harbour a five-year accumulation of deposited atoms whose isotopic composition will offer clues as to the origin of the dust. But Zinner is worried that some of his germanium sandwiches, placed in trays at the leading edge of the LDEF, will have been poisoned or damaged by the long exposure to atmospheric oxygen, in which case the germanium will contain an inseparable mix of terrestrial and extraterrestrial atoms.

But the unexpectedly long exposure of LDEF to the elements of space should also bring some rewards. Many of the experiments on board were designed to assess the damage suffered by a variety of

materials on long exposure to dust, cosmic rays and meteorites, and the body of the LDEF canister itself will be examined closely for pitting and other ill-effects. This whole investigation will add greatly to the sparse body of knowledge on the durability of man-made structures in space, and should provide food for thought for the engineers now designing the space station.

One final LDEF experiment will give schoolchildren across the United States the chance to participate in a little space research. The George W. Park Seed Company of Greenwood, South Carolina, provided 12.5 million tomato seeds in packages loaded on to the LDEF for the Space Exposed Experiments Developed for Students (SEEDS), and set aside another 12.5 million which have been maintained in a sealed environment on the ground since April 1984. The tomato seeds will be divided up into kits of 50 exposed and 50 control seeds, and will be distributed to 250,000 schools as well as to some universities. Students are then free to investigate the effects of space exposure how they choose; young schoolchildren will be able to compare germination rates and times, while university students can look for genetic changes. **David Lindley**

RESEARCH COUNCILS

NERC on the up

London

A "RESURGENCE" in the fortunes of the UK Natural Environment Research Council (NERC) after years of financial attrition, is the optimistic message of the council's report for the year ending 31 March 1989. At a press conference to publicize the report, NERC chairman Professor John Knill described how a 20 per cent rise in government funding over that for 1987–88 reflected "a dramatic change in public and political attitudes to environmental issues".

But the increase in resources available to NERC since the science budget announced in November 1988 did not prevent a 7 per cent cut in staff numbers over the year, a hangover from earlier financial problems. This year's science budget has yet to be allocated among the research councils.

The report also comments on proposals to reorganize the administration and funding of UK environmental research. NERC is opposed to a full merger with the Agriculture and Food Research Council (AFRC), first suggested by the House of Lords select committee on science and technology in October 1988. Nevertheless, there is "considerable logic in a partial merger between AFRC and NERC", perhaps within a division of a single National Research Council. Peter Aldhous

technology. The decision on patent lifetime will not rest with WIPO, however, but with GATT (General Agreement on Tariffs and Trade), which will be considering harmonizing the term of patent protection in discussions of intellectual property rights next year.

Developing nations will be anxious for terms that recognize their special circumstances — the need to have access to sophisticated drugs at reasonable prices, for example. They intend to meet in Geneva before the June WIPO meeting in order to thrash out a common position on the treaty.

Christine McGourty

AIDS

East Berlin risks grows

Munich

TANGERINES and tape recorders are not all that East Germans are bringing back from their trips to West Berlin. Unless East German citizens take precautions, AIDS may be the next big import from the capitalist West. That is the view that emerged when East German public health official Niels Sönnichsen visited an AIDS self-help group in West Berlin.

Sönnichsen, who is at the East German Ministry of Health, and his counterparts in West Berlin have begun an information and condom distribution campaign. The campaign is unprecedented in East Germany, where AIDS was a taboo subject until just a few weeks ago. It is aimed at homosexual men, the only group currently at high risk for AIDS in East Germany. The main message: practise safe sex while in West Berlin.

With 16 confirmed cases of AIDS and seven deaths, East Germany has always been one of the most AIDS-free countries in Europe. By contrast, West Berlin, a city of about 2 million, has had 882 AIDS cases and 349 deaths, according to the West German Federal Health Office. The number of AIDS virus carriers in West Berlin is estimated to be between 5,000 and 10,000. East Germany, with a population of more than 16 million, is thought to have 200 to 400 carriers.

But many of the West Berliners infected have been intravenous drug users, as many as 40 per cent of whom are thought to carry the virus. As there is virtually no drug trade in East Germany, only homosexuals are thought to be at risk there.

A self-help group, Berliner AIDS-Hilfe, has distributed condoms at homosexual meeting places in West Berlin since the wall was opened. The next step, said spokeswoman Birgit Thomas-Schön, is to give doctors in East Berlin training in diagnosis and counselling. But AIDS cannot be stopped at the border, says Meinrad Koch, head of the AIDS centre of the Federal Health Office in West Berlin.

Steven Dickman

Journals and databanks

SIR—Recent discussions in these pages ("Who's hiding the primary data?" *Nature* 341, 94; 1989 and "Making good databanks better" 341, 277; 1989) have praised the goals of the biological databases, pointed out some problems with them, but rejected a role for journals in ensuring their completeness. While we welcome your enthusiasm for our goals, the arguments against the role for journals fail to take account of the facts.

Insistence on deposition of data is seen by you as an unreasonable burden on authors: journals should not be watchdogs; there should be as few obstacles between the author and publication as possible; political, economic and technical restrictions render access to the databases patchy, and it is unreasonable to insist that scientists contribute to a resource that serves them only poorly. Arguments about the workability (there are so many databases nowadays) and enforceability of deposition schemes are also raised.

That journals are watchdogs is already a fact — non-trivial publication standards and review procedures are already in place to ensure the scientific integrity of what they publish. That these procedures are an obstacle between the author and publication does not, of course, justify the creation of others, but the deposition of sequence data is a necessary part of ensuring integrity. It is now common for articles to discuss sequences or structures not actually presented, and even presented sequences would be more accessible in a computer database. Readers cannot assess conclusions based on data they cannot see. Your leading article "Making good databanks better" even stresses that journals should ask for and make available such supporting data as are appro-

priate. What better way is there to do that than through the centralized databases?

That access to the databases is uneven is true. Any implication that we complacently direct our efforts to the service of a high-tech subcommunity is not. For some less well equipped users, we produce tape formats long-since obsolete. Our CD-ROM is targeted for users in low-investment computer environments. It also contains search software that may help to obviate the need to purchase expensive third-party products.

Nonetheless, inequalities exist: but nor are journals, libraries and scientific equipment equally accessible to all. The decision not to include the Soviet Union in the present tripartite nucleotide sequence database collaboration was based purely on a notion of diminishing returns with increasing numbers of collaborators. We routinely exchange data with them.

The databases, again like journals, libraries and scientific equipment, are as accessible to commercial organizations as to academic researchers. Publication of scientific findings (in databases or journals) renders them available for exploration. For the sequence database, this is no bad thing. The development of software tools for exploring genome data is in its infancy. Maintaining the complete data collections in the public domain allows any group — commercial or academic — to carry out research in this area and promotes a diversity of approaches appropriate at this stage.

The workability and enforceability of our data deposition systems is no longer a matter of debate, a number of journals already operate such systems with total compliance, and our feedback from editors and authors is positive. Also, some attempt to minimize the problems of dealing with multiple databanks is being made. The United States, Japan and Europe all have nucleotide and protein sequence databases (incidentally the DNA Database of Japan is at the National Institute of Genetics in Mishima, not the Riken Laboratory as you suggest). These six sequence databases have agreed to deal with journals in a coordinated manner.

Insofar as the databases are pleading for data, they are reflecting the demands of the scientific community. It is the users — publishing scientists themselves — who need the data. We do all in our power to ensure the completeness of our collections, but the task is enormous. Without the support of the researchers at large, we will serve them poorly. For unpublished, possibly confidential data, deposition can be urged. For published data, our goals are compatible with those of the journals. Network access to our databases has been

possible since June 1988; it is not, as you suggest, a thing of the future. Readers of journals enforcing deposition can typically access the data on the day they receive the journal. The two information resources work well together.

On receipt of reasonably documented nucleotide sequence submissions, we issue accession numbers within one week. They are proof of deposition and an unchanging pointer to the data. We feel it would be appropriate for *Nature* to insist that such numbers be presented at some time in the publication process.

GRAHAM CAMERON

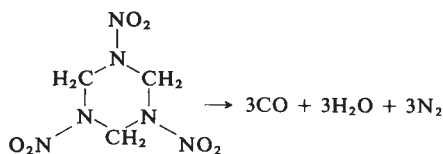
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How not to defeat terrorism

SIR—Robert Michaels' suggestion (*Nature* 342, 336; 1989) that the problem of terrorist bombings of airplanes might be solved by the exclusion of oxygen from the cargo holds of aircraft is ingenious but I am afraid it will not work. Modern molecular explosives, such as TNT, PETN and RDX, do not require oxygen for their detonation but instead contain both oxidant and fuel within the same molecule. For example, the military explosive RDX decomposes on detonation according to the following scheme:



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SIR—Robert A. Michaels (*Nature* 342, 336; 1989) proposes to suffocate terrorist bombs by flushing aircraft luggage bins with nitrogen. DREADCO's torpedo experts assure me that explosives are immune from suffocation. They can explode quite happily in nitrogen, or indeed under water. Human beings, however, are not so self-contained. Michaels' system would merely expose the hapless passengers to a new risk — that of suffocation from nitrogen-leaks. On the bright side, however, it would certainly discourage smokers.

DAEDALUS

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

MS processing

SIR—*Nature* has done a service to authors in an earlier issue this year by publishing the median times in which it accomplishes each stage of manuscript processing. I hope that other journals will take up the same practice. What would be desirable is that all journals not only report on this matter periodically as *Nature* proposes to do, but also reprint the essential statistics at last compilation on the same page or pages where they give their instructions to authors, page charges, reprint prices and so on. A would-be author could then easily find the information when he especially needs it: that is, when considering the advantages and disadvantages of submitting to a particular journal.

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Dangerous chemicals

SIR—Wahlström¹ recognizes the ecological and health problems caused by a great number of existing chemicals and suggests a phasing-out procedure for a 'sunset' list. West German activities in this area may be relevant.

In 1982, an Advisory Committee on Existing Chemicals of Environmental Relevance (BUA), consisting of representatives from science, industry and government, was established at the Society of German Chemists (GDCh). Under the German Chemicals Act, existing chemicals must be reported to the competent authority if they exhibit properties indicating they may be hazardous, either alone or in combination with other chemicals (§ 4(6) ChemG). The goal of that committee is to seek appropriate solutions for dealing with chemicals relevant to health and to the environment by the adoption of voluntary measures. The committee selects and examines existing chemicals for environmental or health purposes as authorized by the German Chemicals Act, using exclusively scientific criteria.

The estimated number of about 100,000 chemicals in the European Inventory of Existing Commercial Substances (EINECS) prevents selection on the basis of strictly quantitative criteria. Even if the necessary resources were available, reliable data for a quantitative evaluation are lacking in most cases.

On the basis of the limited data available, a pragmatic approach has been developed for selection. To this end, a list of chemicals was compiled from various priority lists: the presence of these chemicals in the environment has been proved, or is highly probable; they are industrially important, or are manufactured in large quantities. Approximately 4,500 chemicals were selected in this way, of which about 1,000 were selected because of their occurrence in the environment and their industrial relevance.

After elimination of chemicals subject to special legal regulations, materials of natural origin, inorganic chemicals and chemicals that are unstable in the environment, 512 substances remained. For these substances, data were collected according to eight selection criteria covering environmental exposure and effects. These data were scored in a scoring procedure for selection purposes. The result was a list of 60 chemicals that may be of environmental relevance and which consequently should be treated with priority².

In continuation of this work, a second list of 75 environmentally relevant chemicals was published³. There, the production volume of these chemicals served as an additional criterion. The second list is

associated with considerable uncertainty because of the partial incompleteness of the data and because their scientific quality could not be checked individually in all cases. This means that, as with the chemicals on the first list, the second list may also include substances that are not hazardous to the environment, and also that some environmentally hazardous chemicals have been omitted. Consequently, a conclusive evaluation is feasible only if all available data have been collected in separate substance reports and if data that may be lacking have been obtained by appropriate testing.

Meanwhile, about 40 such individual substance reports have been published and about 80 reports are being discussed or about to be published. The importance of these substance reports can be illustrated by an example. In the case of pentachlorophenol (PCP), production in West Germany has been shut down voluntarily as a consequence of the report because PCP is considered as a major source of dioxins in the environment⁴. It is disappointing that companies in other countries have not followed these recommendations, but have even increased their production. As long as non-ecological behaviour leading to more profit governs our decisions, no solution to our environmental problems can be expected.

A third priority list is in the process of being evaluated by an *ad hoc* working group of the advisory committee. In addition, regular scientific symposia provide a platform for the presentation and discussion of the results to the scientific community and the public. Two such symposia have so far been organized under the auspices of the Society of German Chemists.

In conclusion, the commendable five-step phasing-out procedure suggested by Wahlström has already been realized on a national basis in the West Germany. The selection criteria and priority lists developed by the Advisory Committee on Existing Chemicals of Environmental Relevance could also serve as an international model.

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2. GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance (BUA) *Existing Chemicals of Environmental Relevance, Criteria and List of Chemicals* (VCH, Weinheim and New York, 1989).
3. GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance (BUA) *Existing Chemicals of Environmental Relevance II, Selection Criteria and Second Priority List* (VCH, Weinheim and New York, 1989).
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Counting partners

SIR—It is true that the average (lifetime) number of heterosexual partners per person should be the same for men and women (*Nature* **342**, 12; 1989), but this will be apparent only if the total population is known. Concentration of promiscuity in a small minority of women will cause most samples to show a wide difference between the sexes.

To take a hypothetical example: suppose a population of 10,000 men and 10,000 women in which 9,990 of the women and 1,000 of the men have been faithful to their partners, and in which the remaining 10 women have earned their living by making themselves available to each and every one of the remaining 9,000 men; almost all samples will show the average number of partners as one for the women and ten for the men.

It should not be supposed that most informants have lied but that researchers have had difficulty in finding sufficient numbers of highly promiscuous women. All the more reason for expanding the research!

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Embryo research

SIR—The dilemma facing the British government in implementing the Warnock recommendations¹ is that, if experimentation with or destruction of artificially fertilized embryos is prohibited after 14 days, anti-abortionists could demand the same protection for naturally fertilized embryos. One solution is to scrap the arbitrary 14-day limit.

The German government has chosen a second solution. It is about to legislate against any embryo research which is not for that embryo's own benefit. In so doing, the German government is acting on the Nuremberg Codex developed after the Nazi war-crimes trials². Should this not give us pause?

Certain German scientists and doctors rationalized, supported and even participated in the Holocaust^{3,4}. This fact is now universally abhorred. But how many of us would not have been among them, given normal concerns for personal and family security and also career pressures? If this is so, we must now ask how many of today's scientific lobby for embryo research are free of such concerns and pressures. If not thus free, may not today's lobby be equally mistaken?

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The overdose of drugs in Japan

Masanori Fukushima

The Japanese consume enormous amounts of drugs on prescription, some of which are not legally available elsewhere in the world. The reason is a defective national system for drug approval and dispensing.

THE Japanese are the world's highest spenders on prescription drugs. Among the top-selling drugs are anti-cancer agents peculiar to Japan that are of questionable efficacy and yet have sales of hundreds of millions of dollars each year, far more than the sales of anti-cancer drugs in other developed nations. Moreover, several of the drugs sold only in Japan have serious side effects. Justification of these contentions is to be found in Tables 1–3.

What has caused this worrying situation? And how can drugs be million-dollar sellers in Japan and yet not sell elsewhere in the world? The answer is that there is a whole series of defects in Japan's drug-approval process and in the system for dispensing approved drugs.

At first sight, Japan's system of clinical trials resembles that of some other countries. In principle, drugs must pass through three phases of clinical trials as, for example, in the United States. Phase 1 is to check a drug's safety in humans; phase 2 is to assess its therapeutic index (the response rate and the severity of side effects) in selected populations of patients for whom the drug is intended; and phase 3 is to determine whether the new treatment is better than existing ones. In the case of anti-cancer drugs, however, once a drug has passed phase 2 it can be widely prescribed throughout Japan. The phase 3 clinical trials in effect become part of the pharmaceutical company's marketing strategy. Further, they do not meet the rigorous requirements of such trials elsewhere in the world — they tend to assess effect only (reduction in tumour size, for example), which is measured during phase 2 trials, rather than benefit (risk-to-benefit, and cost-to-benefit), which is measured during phase 3. So drugs which have some effect but are of little proven benefit can reach the market.

A further disturbing feature is that patients are often given drugs under trial without being asked for their informed consent — that is, without it being explained to them that the drug is experimental, or obtaining their signature on a specific document outlining certain details about the particular study and acknowledging the patient's willingness to participate in it. In the United States and other developed countries, patients involved in clinical trials must fill out and sign detailed consent forms. Thus many drugs that sell well in Japan cannot be sold elsewhere

because satisfactory phase 3 trials have not been carried out.

In contrast with conditions in countries such as Britain, where doctors are not allowed to sell drugs, nearly all Japanese general practitioners and hospitals have their own pharmacies for outpatients. Every two years, the Ministry of Health and Welfare (MHW) sets 'official' prices for all drugs, and these prices are supposed

TABLE 1 Sales of pharmaceuticals for human consumption, 1987

Country	Sales (\$ × 10 ⁹)	Expenditure per capita (\$)
United States	28.965	109
Japan	22.698	166
West Germany	7.606	136
France	6.754	128
Italy	5.962	109
Britain	2.596	51
Spain	2.053	51
Netherlands	0.964	57
Belgium	0.870	114

Source: DIALOG PTS PROMT.

to determine the charges to patients and the national health insurance system. But pharmaceutical companies, as a matter of standard marketing strategy, offer the drugs to hospitals at a discount. The permitted discount is 10 per cent, but currently discounts are typically 20–30 per cent or more, particularly when several companies are competing for a share of the market. There is thus a tremendous financial incentive for doctors to prescribe drugs excessively and unnecessarily because they make a profit on every sale. Those new to Japan are often surprised when they go to a doctor with a simple cold and are given an injection of vitamin B and are loaded with drugs to take away. Another odd feature is that drugs are unlabelled, so patients do not know what they are taking.

Apart from being a massive drain on the national health system and on the pockets

of patients, over-prescription and over-use of drugs has had some disastrous consequences. A classic example is that of blood products. In the early 1980s Japanese pharmaceutical companies began to import large amounts of cheap blood products from the United States, selling them to hospitals and doctors at well below the official price. By 1985 annual per capita blood-product consumption in Japan had risen to about three times that of Britain and France¹. Coincident with the surge in imports, AIDS was introduced to Japan in non-heat-treated blood coagulants used for the treatment of haemophiliacs. Japan has about 1,000 carriers of AIDS, more than 90 per cent of whom are haemophiliacs.

Other examples are the anti-cancer drugs PS-K (Krestin), OK-432 (Picibanil) and Tegafur, all available only in Japan. Research on Tegafur has been abandoned elsewhere in the world², but in Japan Tegafur, together with Tegafur uracil, has annual sales of \$280 million (Table 2). PS-K and OK-432 are widely prescribed — PS-K for cancers of the digestive organs (stomach, oesophagus, colon and rectum), lung and breast; OK-432 for cancers of digestive organs (stomach, liver, biliary tract, colon and rectum), head, neck, thyroid and lung. Total sales of these and other anti-cancer drugs peculiar to Japan are about \$1,000 million per year. But have these enormous expenditures resulted in an increased number of lives saved? The simple answer is probably no.

Since the mid-1960s, survival rates for various cancers have steadily improved, but comparison of the rates with those of other countries where these drugs are not available³ shows no evidence of increased survival in Japan (Table 4). The exception

TABLE 2 Top-selling anti-cancer drugs in Japan and the United States, 1987

Japan			United States		
Drug	Sales (\$ × 10 ⁸)*	Percentage of market share	Drug	Sales (\$ × 10 ⁸)	Percentage of market share
1. PS-K ^{†‡}	515	25.2	Doxorubicin	86	16.3
2. OK-432 ^{†‡}	275	13.4	Cisplatin	79	15.0
3. Tegafur uracil [§]	255	12.5	Tamoxifen citrate	68	12.9
4. 5-Fluorouracil	150	7.3	Etoposide	52	9.8
5. Tegafur [§]	145	7.1	Cyclophosphamide	31	5.9
6. Tamoxifen citrate	90	4.4	Methotrexate	29	5.5
7. Interferon- β	58	2.8	Megestrol acetate	27	5.1
8. Lentinan [§]	45	2.2	Mitomycin C	25	4.7
9. Carmofur [§]	38	1.9	Bleomycin	20	3.8
10. Estramustine phosphate sodium	36	1.8	Vincristine sulphate	18	3.4

Source: Estimate of Pharmaceutical Industries.

*¥144 = \$1. [†]Kinds of biological response modifiers.

[‡]Listed as an investigational drug in *AMA Drug Evaluation*, 6th edn (Saunders, Philadelphia, 1986).

[§]There is no entry for these drugs in standard manuals, meaning they are not recognized drugs in the developed nations.

is stomach cancer, but the higher survival rate of these patients is most likely to be due to early diagnosis in Japan.

PS-K is of very doubtful efficacy. Studies during clinical trials seem to show some evidence of increased survival among cancer patients⁴⁻⁶, but most of the research was published in company-financed or company-related journals; the only report in an international journal is a review of the Japanese work⁷. Although comparative studies were carried out in these clinical trials, they were severely flawed. For phase 3 studies, for example, disqualifications are a source of potential bias⁸. In the trials the disqualification rates for major end-points were 18 to 36 per cent, greater than the magnitude of the differences in outcomes tested. Furthermore, there was no assessment of whether patients complied with the requirements of the protocol, no description of procedures to ensure that the data are reliable and no analysis of whether there were significant deviations in findings from one institution to another. Also, the results have not yet been reproduced.

The MHW has reinvestigated the efficacy of OK-432 and PS-K, and a decision to restrict their use to combinations with other chemotherapeutic agents is imminent, according to Japanese newspaper reports. But as the drugs are usually used in combination with other agents, this is unlikely to have much effect on prescriptions and sales.

So why is PS-K so widely used despite its questionable efficacy? One reason is that it has no side effects. In Japan, doctors tend not to tell their patients if they have cancer, reasoning that the shock might kill them. PS-K is thus a handy drug to prescribe as it is taken orally and has none of the tell-tale side effects (such as hair loss) of anti-cancer drugs used in the West. Another reason is that most Japanese doctors are unfamiliar with the discipline of medical oncology. Finally, once a drug has passed clinical trials, the MHW tends to approve it for a wide range of related diseases without assessing its therapeutic efficacy or clinical usefulness for each disease. MHW has approved the use of interferon- α for kidney cancer and multiple melanoma, and that of interferon- β for glioma and melanoma, for example. But although interferon can produce some response in tumours of these cancers, at present there is no evidence of an increased survival rate among patients. In the United States, the FDA has approved interferon- α only for hairy-cell leukaemia and melanoma.

To understand why there are so many dubious drugs on the Japanese market, one has to understand the unhealthy close relationship that exists between doctors, pharmaceutical companies and the MHW. Data presented to the MHW for approval are in mainly in the form of

TABLE 3 Documented adverse reactions to drugs sold only in Japan

Date disclosed	Drug	Date of approval	Side effects	Victims (deaths)
Sep. 1987	Peplomycin	Mar. 81	Interstitial pneumonia	? (8)
Jul. 1988	Carmofur	Sep. 81	Leukoencephalopathy	44(?)
Dec. 1988	Aspirin DL-lysine	Jun. 82	Shock	26(5)
Feb. 1989	Calcium hopatenate	Nov. 83	Severe encephalopathy	47(11)
Jun. 1989	Medroxyprogesterone acetate, high dose*	May 87	Myocardial, brain infarction	33(11)
Aug. 1989	OK-432	Oct. 75	Delayed type shock	13(3)

*This drug is sold in low doses in the United States and Europe.

TABLE 4 Comparison of five-year relative survival rates*

Sex and site of cancer	Osaka 1978-80	United States 1973-79		Britain 1971-73	Finland 1972-75
		White	Black		
Male					
Stomach	36.4	12	13	7.4	9.5
Colon	39.2	47	41	29.6	32.3
Lung	9.8	10	8	7.8	7.4
Female					
Stomach	28.4	14	16	7.3	8.3
Colon	31.0	49	47	29.4	31.2
Lung	9.6	14	11	7.0	9.8
Breast	69.1	72	60	56.8	57.6

Source: ref. 3. *Figures are percentages.

unpublished results or of reports published in Japanese in company-financed or company-related journals. Those in charge of clinical trials often set up their own foundations, funded by the pharmaceutical companies, for which they organize clinical trials. And often some heads of clinical trials also belong to the drug-approval committee. Thus a data-producing mechanism by the company for the company is inherent in the system.

What can be done? As some first steps I suggest the following.

- Only data published in leading international journals should be acceptable, and the work of more than one group of researchers must be available for evaluation; claims for drug effectiveness appearing in Japanese journals (except scientific society journals) should be discounted, as should unpublished results.

- The published evidence should be checked by referring to the individual patient's chart, and the data on the drug's toxicity and adverse effects should be examined.

- The MHW staff must be able to judge whether or not the papers are scientifically sound. The diagnostic criterion concerned must be checked and the results strictly interpreted according to the backgrounds of the patients concerned.

- In arriving at a decision, MHW staff must carefully analyse the risk-to-benefit, cost-to-benefit and cost-to-effectiveness factors, and should address questions such as what sort of benefits the drug will have. Needless to say, the members of the approval committee and MHW staff must be scientifically qualified (at present many are not). MHW should also enforce "good clinical practice" (ref. 9) on clinical trials, direct doctors to obtain informed consent properly, encourage them to pursue well-designed and controlled phase 3 studies, and assess adverse drug reactions.

- To cut down on Japan's wasteful consumption of drugs, pharmacies run by hospitals and individual doctors should be abolished and replaced with independent outlets. MHW is moving in this direction

(a process called *bungyo*, meaning divide) but very little progress has been made.

Many of the problems I have described stem from the poor quality of science practised by Japanese clinicians, which in turn stems from the inability of most of them to read the foreign literature. But the problems are more deep-rooted than just language. In Japan there is little appreciation of the value of individual thought or philosophy which sustains science and is the spring of new ideas elsewhere — the lack of informed consent in medical practice is just one manifestation of that failing.

It seems that things have not changed much since 1902, when Dr Rintaro Mori (Ougai Mori), pointed out the Japanese were unable to sustain science but were very enthusiastic about eating its fruits. This attitude partly explains why Japanese research tends to be mostly method-orientated and unoriginal, and why some scientific fields created by other developed nations — clinical pharmacology and medical oncology, for example — are not established here. Radical, structural changes are needed if Japan's drug-approval system is to work for the benefit of the public and not for that of the pharmaceutical companies. □

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Reflections on the discovery of fission

Rudolf Peierls

Sir Rudolf Peierls adds a personal postscript to a year that marked the fiftieth anniversary of a discovery that helped shape the twentieth century.

THE publication of the paper by Hahn and Strassmann¹ in *Naturwissenschaften* in February 1939, 50 years ago, is rightly considered the essential step in the discovery of fission, although it was preceded by several years of experiments. But the background to this discovery lies much further back.

In 1932, the neutron was discovered by James Chadwick. It was immediately clear that it was a constituent of atomic nuclei, and soon it also became clear that nuclei consist of only neutrons and protons; there are no electrons in nuclei. The electrons emitted in beta decay are created in the act of emission. This insight marked the beginning of nuclear physics as we know it today.

Enrico Fermi in Rome was one of the first to realize that neutrons could become an important tool for studying nuclei. He became excited about this prospect and, although he had previously done important work in theoretical physics, he decided in 1933 to become an experimenter. By 1934 he had built his equipment and started experimental work with a group of collaborators.

The only way to generate neutron beams then was to bombard light elements with charged particles, either alpha particles of the kinds emitted from radioactive sources or protons from an accelerator, such as a cyclotron or a high-voltage installation. Rome had no accelerator, so Fermi was compelled to use the laborious method of directing alpha particles at beryllium.

Early observations

Fermi discovered that, in many elements, slow neutrons are more efficient at hitting nuclei than fast neutrons, which makes the experiment easier, provided one interposes between source and target a substance containing hydrogen or other light elements, a 'moderator', in which the neutrons can collide with the light nuclei and lose kinetic energy to them.

Even those early observations were a puzzle for the then rudimentary theory of nuclei. It was then generally assumed that one could treat the passage of a neutron through a nucleus in good approximation as if it were moving in a potential field, which would be the average field of the particles inside the target nucleus. This picture, taken over from atomic theory, would of course have to be corrected for exchange of energy between the neutron

and particular target nucleons, but this was taken to be a small correction.

On this basis, one would have expected the main result of a neutron-nucleus collision to be elastic scattering of the neutron, with an occasional inelastic event leading to excitation of the nucleus, and only very rarely the capture of the neutron with the emission of radiation. But the experiments showed that neutron capture was a very frequent event — for many nuclei, much more probable than scattering.

These findings completely mystified theoretical physicists until the solution was presented² by Bohr in 1936. Briefly, his point was that the constituents of the nucleus are interacting very strongly with each other, and with the incident particle, so that its kinetic energy is shared between many particles, with the result that none of them can escape until enough energy happens to be concentrated on one of them. This means that the neutron must remain in the nucleus for a much longer time than the picture of a single particle in a potential would have predicted and that, during that time, the chance of the emission of a photon is appreciable. In the case of a slow neutron, for which practically the whole excess energy is required for escape, escape becomes a very infrequent event and the emission of radiation dominates.

In most elements, the capture of a neutron produces a nucleus which is beta radioactive. (Artificial beta radioactivity had been discovered by Frédéric and Irène Joliot-Curie in 1933.) For different radioactive nuclei produced in this way, the beta rays have different half-lives, so that it is possible to distinguish the radioactive species formed. By chemical separation of the activated target, one can discover the chemical nature of the radioactive nuclei produced.

After establishing the principles, Fermi and his group bombarded with neutrons all elements they could get hold of, practically the whole periodic system of elements. This exploration produced a large amount of data, from which one could learn much about the properties of nuclei. A particularly interesting case was uranium, because that was the heaviest nucleus and at the end of the periodic system. If uranium captured a neutron it would become heavier. Because the number of neutrons in it would then be too large for the number of protons, it would probably increase its charge by emitting a

beta electron and would thus become a new element, not existing in nature — a 'transuranic' element. The experiments on uranium were therefore watched with particular interest.

But the results were very unexpected. Instead of one, or a few, half-lives, the decay curves showed there were several, so many that it was not easy to disentangle the curves and find how many there were. How could neutron capture produce so many different species of nuclei from one element? This was a puzzle that many physicists tried to solve. Could a nucleus be found in many different states, without quickly being transformed to the most stable of them? Examples of such 'isomeric states' were known, though not in such large numbers. Niels Bohr was one of the theoreticians who tried to invoke such isomeric states to explain the behaviour of the alleged 'transuranic elements'.

Some answers

A different answer was proposed by a chemist, Ida Noddack-Tacke, as early as 1934³. She started from the fact that in his attempt to identify the chemical nature of the active nuclei, Fermi had looked only at elements of atomic number Z above 82, because none of the reactions known or imagined in nuclear physics could have resulted in a lower Z . Noddack-Tacke suggested that perhaps the uranium nucleus could have split into several sizeable pieces, whose atomic number then would be much smaller. So the experimenters should therefore look for activities following the chemical behaviour of much lighter atoms.

Nobody seems to have taken this suggestion seriously. Fermi (to whom the author presumably sent a copy of her paper) said such a capture of the neutron would not be sufficient to overcome the forces holding the nucleus together. Probably others who saw the paper also had difficulty in accepting the idea of a reaction that was so different from what had ever been seen.

Efforts to resolve the puzzle continued for many years. In Berlin, Hahn, Lise Meitner and Strassmann did painstaking chemical analyses to identify the chemical properties of the radioactive nuclei created. Joliot-Curie and Pavel Savich analysed one particular activity very carefully and found that it appeared to belong to an element with chemical properties indistinguishable from lanthanum, which

is in the middle of the periodic table⁴.

This lead was followed by Hahn and Strassmann (Meitner had by this time had to leave Germany because of her Jewish origin). They found more and more convincing evidence that one particular activity behaves chemically as if it was due to an isotope of barium. The chemical properties of barium are rather similar to those of radium and to those expected of a transuranic element, so that to discriminate between them required very sophisticated analytical techniques. But a paper published in *Naturwissenschaften* on 6 January 1939 by Hahn and Strassmann⁵ said that, as chemists, they must conclude that the activities were due to isotopes of barium, lanthanum and cerium, but that, as physicists, they were not yet ready to accept a conclusion that ran counter to all experience of nuclear physics. But in their paper of 10 February¹, they finally stated that the conclusion was inescapable that the uranium nucleus had split in two.

Even before the first paper of Hahn and Strassmann had appeared, Hahn had reported their conclusion in a letter to Meitner in Sweden, and she discussed this surprising finding with her nephew, O. R. Frisch, who was visiting her at the time. Between them, they saw that one could understand the splitting of the nucleus as a process in which the electrical repulsion between different parts of the nucleus overcame the overall effect of the attractive forces between protons and neutrons in the nucleus, which hold the nucleus together and which may be likened to the surface tension which is holding together a liquid drop.

The impact and capture of a neutron, which liberated an energy of about 7 MeV (the binding energy of a neutron in a nucleus) causes oscillations in the shape of the nucleus, from spherical to oblate and prolate forms and back. The prolate form is favoured by the electrical repulsion and, if the shape becomes sufficiently prolate, the electrical repulsion can result in the nucleus splitting in two. The kinetic energy of the fragments would equal the potential energy involved in their repulsion, which Meitner and Frisch could estimate, and which agreed with the difference between the binding energy of the uranium nucleus and that of nuclei of roughly half the atomic weight.

Meitner and Frisch wrote a letter to *Nature*⁶ with their explanation. They introduced the name 'fission' for the process. Frisch chose the name because it was used in biology for the division of a cell, which seemed a suitable analogy.

Returning to Copenhagen, Frisch reported the idea to Bohr, who saw the point immediately and exclaimed "Oh, what idiots we all have been!". Bohr was about to leave on a journey to the United States, and he promised Frisch he would

not talk about this development before the notes by Hahn and Strassman and by Frisch and Meitner were published, so as not to compromise their priority.

George Placzek, a very knowledgeable theoretician, then also in Copenhagen, did not believe the story, and challenged Frisch to demonstrate the fission fragments directly. Frisch immediately set up an experiment, which he later said was really quite easy, and in two days the fragments showed up in his apparatus⁷.

It is now clear that Fermi had just missed seeing the fragments by a similar method several years earlier. He had set

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Working it out — Niels Bohr at Princeton.

up a counter near a uranium target being irradiated with neutrons. But because he feared that the alpha particles from the uranium would saturate his counter, he covered it with a foil that would stop the alpha particles and which also stopped the fission fragments. A less careful experiment would have shown Fermi the intense ionization caused by the fragments, which would have stood out clearly above the alpha-particle background.

Meanwhile, Bohr had arrived in Princeton. He was accompanied by his collaborator Léon Rosenfeld, who preceded him to Princeton while Bohr spent a few days in New York. Unfortunately, Bohr had neglected to tell Rosenfeld that he intended not to tell people about fission, so Rosenfeld innocently talked about it at Princeton, causing a sensation among physicists. It was the main item in a conference at Washington on 26 January, at which Bohr talked about the experiments of Hahn and Strassmann and the explanation by Meitner and Frisch. He was embarrassed about the leak, but the news was out, and he could at least stress the credit due to these authors. News of Frisch's experiment had not as yet reached him.

Several US physicists immediately carried out experiments to demonstrate the fission fragments, using methods similar to that of Frisch, whose own experiment had been completed but which had not yet been reported in the United States. Energetic investigations of the new phenomenon began in many laboratories. One

puzzling finding was that in uranium, and also in thorium which had also been found to undergo fission after the capture of neutrons, fission was not the only outcome: transuranic elements seemed also to be produced.

This gave Bohr the clue for a fine piece of detective work. Uranium has several isotopes, of which the most common (of atomic weight 238) constitutes more than 99 per cent of natural uranium, and the lighter isotope (atomic weight 235), constitutes only about 0.7 per cent. Bohr came to the conclusion that ²³⁸U and thorium split only when bombarded with fast neutrons, and that slower neutrons, in particular the very effective thermal neutrons, caused fission only in the less common ²³⁵U (ref. 8). Some people, notably Fermi, did not accept Bohr's argument, and remained sceptical until a year later small samples enriched in the lighter isotope became available, and direct measurements proved Bohr right⁹. Bohr continued working on the theory of fission, and, in collaboration with John Wheeler, he produced a treatment of many aspects of this problem¹⁰, which became a classic.

One implication of the discovery immediately aroused speculation among physicists: the possibility of releasing some of the energy stored in the atomic nuclei. It had been known for a long time that there are nuclear reactions in which energy is released, and in this respect fission was just another process, though with an extremely large energy release.

I heard Lev Landau explain the problem with great clarity in 1934. He pointed out that the then known reactions were initiated by the impact of a charged particle on a nucleus. As we cannot aim our bombardment at a nucleus, the vast majority of projectiles will get stopped by interaction with the atoms of the medium without ever hitting a nucleus, so that the energy wasted far exceeds that gained from the few direct hits. Neutrons are different, Landau said, because they are not stopped, and they go on until they hit a nucleus. But the only way we have now (in 1934) of producing neutrons is by charged-particle bombardment, and so we are back with the same problem. But if someone finds a reaction caused by neutrons which produces several secondary neutrons, you are all set. As early as 1934, Landau was of course referring to a chain reaction, in which the neutrons of each generation produce a larger number in the next generation, so that the total number grows exponentially and, unless other processes intervene, rapidly reaches macroscopic magnitude.

Leo Szilard also speculated about the possibility of a chain reaction, and even took out a patent on it, but without knowing how to implement it. He had at one time the misguided idea of a chain reaction in beryllium. Probably others

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saw the situation more or less as Landau saw it, but nobody published such speculations.

So it was natural that after the discovery of fission the question arose whether the fission was accompanied by the emission of secondary neutrons. The first positive answer to this question was reported by Joliot's group in Paris¹¹. Their estimate of the number of neutrons per fission was somewhat high. More accurate measurements were done a little later by Fermi's group at Columbia University, New York¹².

A chain reaction requires that, of the neutrons released in the fission of one nucleus, more than one neutron should cause the fission of another nucleus. This requires that the loss of neutrons by other processes be below a certain limit; if this condition is met, there will be a critical size, because in too small an amount of material, the proportion of neutrons leaving the body before hitting a uranium nucleus would be too large.

By now, the possibility of a chain reaction had become more real, but there were still difficulties. As Bohr had pointed out, the abundant isotope ^{238}U can be split only by fast neutrons with energy above a threshold now known to be about 1 MeV. Only a fraction of the neutrons created in the fission process have such high energies, and they tend to lose energy by inelastic collisions.

The large effect of slow neutrons on ^{235}U suggested slowing down the fission neutrons by adding to the uranium a moderator, that is, a light element. The neutrons collide with the atoms of the moderator and thereby lose energy. But the moderator also absorbs some neutrons, and for most light elements the absorption is great enough to reduce the average number of neutrons per fission to less than unity, thus making a chain reaction impossible. Moderators that have been used successfully with natural uranium are carbon, in the form of graphite, and heavy water (D_2O).

Fermi succeeded in making the first chain reaction by using a graphite moderator. For this, commercially pure graphite would not do because certain impurities can absorb too many neutrons even if they are present in concentrations undetectable by chemical means. Fermi insisted on further purification, in the belief that there were still excessive impurities, and indeed the absorption of neutrons was reduced below the critical level. With this purified graphite the first chain reaction was produced by Fermi's group in Chicago on 2 December 1942.

The success of this experiment opened the way to the release of nuclear energy on a macroscopic scale. The most obvious application was to power generation. This would consist of reactors working in principle like Fermi's first experimental one,

but with arrangements for heat transfer, which allowed the heat generated in the uranium to be passed to steam generators or other devices for driving machinery. There were of course many practical problems to be overcome, but they were technological rather than nuclear physics problems.

Another application was to the production of radioactive isotopes, which are useful in many areas of research, and for medical diagnosis and treatment. Nuclear reactors are an abundant source of neutrons, which can be used to bombard various targets and to produce in them a great variety of radioactive species. But at the time all these possibilities were for the future. The world was at war, and interest was concentrated on the third possible application, the nuclear bomb.

Bohr had pointed out that a reactor could not produce a violent explosion. The reactor depends on slow neutrons, which have to travel considerable distances before they cause further fission, so that the development of the chain reaction is slow. When the released energy has heated the reactor to a temperature that will melt or vaporize its materials, the expansion and dispersal will stop the chain reaction. The energy released is therefore only of the order of the cohesive energy of the structure, so that the explosion is no more violent than that from chemical explosives.

In 1940, I was discussing such problems in Birmingham with Frisch, and we felt rather reassured by Bohr's argument, which seemed to show that a fission bomb was impossible. Then, one day, Frisch asked me: "Suppose someone gave you a lot of separated ^{235}U , what then?" This seemed an entirely academic question because the separation of isotopes had been achieved only in gram quantities and only for light elements, where the fractional mass difference of the isotopes is much greater than the roughly 1 difference in uranium.

With ^{235}U , every neutron is capable of causing fission, and one does not need to rely on the especially high capture rate for slow neutrons, so one needs no moderator. We could guess at the nuclear cross-sections from Bohr's theory, and I had a formula ready to give the critical size in terms of the cross-sections. We were staggered to find that the critical size, or mass, was only pounds, instead of the tons one intuitively expected after thinking about reactors.

How much energy would be released in such a fast chain reaction? Now the neutrons were 10,000 times faster than the thermal neutrons effective in the reactor, and they had a much shorter distance to travel. Therefore the build-up of the chain reaction would be fast enough to cover an appreciable fraction of the uranium nuclei before the pressure from the heat

developed would disperse the materials and stop the reaction. One would have an explosion of unprecedented power.

It seemed to us that the enormous power of such a weapon would be worth the great effort required to separate the uranium isotopes. This was in 1942, and we feared that Nazi Germany might complete such a weapon first. We knew there was no defence except the threat of retaliation.

We wrote a memorandum to the British authorities, in which we presented our arguments and outlined, as best we could, the implications. We mentioned the fall-out and the inevitability of many civilian casualties in almost any use of such a weapon. We did not foresee the fusion bomb, nor the insane arms race.

These arguments were taken seriously by the British government, and this in turn led to an accelerated pace in setting up a large-scale project in the United States — the Manhattan Project. This succeeded in separating large amounts of ^{235}U , but also followed an alternative approach: some of the ^{238}U nuclei capture a neutron to produce ^{239}U which is itself unstable and emits a beta electron to become neptunium-239 (^{239}Np). That, in turn, undergoes rapid beta decay to become plutonium-239 (^{239}Pu), which lives for a long time. It was conjectured from Bohr's theory, and later verified, that in ^{239}Pu , as in ^{235}U , neutrons of any energy would cause fission; it was also weapons material. So another activity of the Manhattan Project was to build large reactors producing plutonium. It is common knowledge that both these methods led to the production of nuclear weapons and that they were used against Japan.

This is not the place to discuss the moral implications of the making and the use of the bomb, or the best way of avoiding nuclear war, or to argue the advantages and dangers of nuclear power. My objective has been to tell the story of the very esoteric work of academic physicists and how in such a short time it developed into applications of enormous military, political and economic importance. □

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Making authors toe the line

The idea that those who wish to contribute to the scientific literature should first satisfy preconditions sends shivers down the spine: are there compromises to be worked out?

DATABASES have come to stay and, like many other institutional innovations, have roused great passions. So much is plain from the manner in which the supporters of particular databanks advocate their virtues — with zeal, sometimes with blind zeal. Only a few weeks ago, in a nice illustration of that genre, one correspondent found himself declaring that “it is a simple matter to require that the author of a sequence paper provides an accession number . . . as a prerequisite to publication” (*Nature* 342, 114; 1989). But is it?

So far as this journal is concerned, most passion centres on the decision that preconditions on the publication of research material are not simple. In particular, it has not hitherto been a precondition of the consideration of a manuscript for publication that nucleotide sequence data (if any) should first have been registered with one of the three branches (in Europe, the United States and Japan) of the international enterprise for compiling a coordinated nucleotide sequence databank. What follows is yet another explanation of what is plainly a contentious issue and of what some may even consider to be a remedy, this time evoked by the letter from Dr Lennart Philipson and his colleagues at Heidelberg (see page 849).

The first need, obscured in many of the arguments conducted in recent months, is that we should acknowledge the virtues of most databases, and of the nucleotide sequence databank in particular. It would be a great public service if there were a generally and uniformly available set of nucleotide sequences to which researchers could refer. Despite operational difficulties (many of them teething troubles) at the three centres, the service they provide is a good first approximation to a uniformly accessible sequence databank. There is good reason to expect that the arrangements now in place will be able to handle the much increased volume of data expected in the next few years. So far, it might be thought, so good. These are some of the reasons why this journal has, for several years, “urged” its authors to send their data to the sequence databanks. So why not go the whole hog, and make that a precondition?

This is not the arcane argument it may seem, but rather an issue of principle touching the relations between journals such as this and their contributors on the one hand and their readers on the other. It is not, of course, absurd, or an infringement

of contributors' rights, that journals should require their contributors to satisfy certain preconditions — so many copies of a manuscript, typed in double-spacing, for example. Their justification is the mutual convenience of journals and their contributors that decisions should be reached quickly and that manuscripts chosen for publication should be as quickly processed. Tacitly, at least, such points are easily agreed.

Difficulties arise when contributors are asked to satisfy conditions that have nothing to do with the content of what they have to say, or the manner in which that is presented. Compliance with the wishes of the databanks is only one of the requirements that might be made of them. Why should not journals also demand, for example, that their contributors should have complied with the law? Would it not make for the tidier administration of regulations on the use of recombinant DNA techniques that the appropriate committees, if relevant, should have been notified and have given their approval? And the same, *mutatis mutandis*, for embryo research, the use of radioactive chemicals in the laboratory or the use of experimental animals in research? While they are at it, why should not journals also take on the administration of national legislation on secrecy?

Not all those who urge this journal to make mandatory the prior submission to databanks of nucleotide sequences would go so far as to applaud all possible innovations along these lines. An earlier correspondent (*Nature* 342, 114; 1989) correctly argued that the interaction between journals and their contributors, usually anxious to appear in print, is a point at which pressure can be effectively applied. But that is all the more reason why journals, rightly fearful of being turned into instruments of law-enforcement, should resist it. And the only simple recipe for the avoidance of the consequences of prior conditions on publication is that there should be none.

Relations between journals and their readers are also important. The business of publication, which is an honourable and socially valuable business, is that of making information generally available. In principle, at least, all readers should be on an equal footing, with an equal chance to make what they can of what happens to be published. That is why this journal has, uniquely among journals with an interest in research, taken the trouble to print

itself at three centres in world (so far). It remains a constant source of disappointment that the West Coast of the United States and Australia should be less quickly served than, say, New York or London.

Readers' appreciation of what they read depends, of course, on circumstances. People somehow in the swim are usually better placed to appreciate the full significance of what they find it interesting to read than those exiled to the sticks. Often it may be that a reader's understanding of a novel concept in a research article will fully flower only when he has talked it over with his or her colleagues, which is another kind of privilege. Most journals prudently acknowledge that these are circumstances they cannot change, much as they might wish to do so. But there are no circumstances in which a journal such as this, founded 120 years ago to bring the record of research to a wide audience, could fall in with the idea that its readers' appreciation of what they read will hang crucially on the accessibility of a databank in Heidelberg, Los Alamos or Mishima.

Accordingly, this journal has determined as follows: we shall continue (as at present) not to require of our contributors that they should have submitted their data (if in any way relevant) to the international databanks before submitting them for publication, but we shall threaten them that, if their articles appear in print and happen to be supported by nucleotide sequences, even if not intended to be published, we shall send those data off to Heidelberg or whichever other centre appears more congenial and also promises to make the data more generally available. We shall also make exactly the same data available to such of our readers who happen to telephone or to write. We shall do that without rancour, but shall aim to give the databanks a good run for their money. From time to time, we shall be willing to listen to people who have special secrets to hide.

This, then, is this journal's proposition: people with a nucleotide sequence to disclose should either disclose it or keep mum. They should not seek the best of both worlds, that in which they get credit for the sequence without saying what it is. Nor, for that matter, should they expect to be able to publish a sequence of nucleotides without being able to guess at what it means. Somewhere between, there must be a happy mean. Curiously, that boils down to an editorial function, not an issue of principle.

John Maddox

Helium line in the Banda arc

Eli A. Silver

A LONG-STANDING enigma in geotectonics is just what melts to produce arc magmas in a subduction zone — where the cold, dense edge of oceanic lithosphere (crust and upper mantle) sinks into the deeper mantle. Can one recognize in the arc lavas differences in material entering the subduction zone? One approach to these questions is to use geochemical markers in the volcanics of island arcs, and a number of such techniques are known¹. Results reported by Hilton and Craig on page 906 of this issue² show that helium isotope ratios, a useful geochemical marker, in island-arc basalts achieve a spatial resolution unequalled by other studies. Simultaneously, they identify remarkable processes occurring at the Sunda-Banda volcanic arc, a subduction zone in the Pacific ocean.

Earlier this year, Poreda and Craig³ reported on a survey of circum-Pacific island arc studies of the ratios (R) of $^3\text{He}/^4\text{He}$ in arc materials relative to those (R_a) in air. (^3He is radiogenic and must be derived from contamination by continental crust.) They discovered that most of the circum-Pacific arcs have ratios R/R_a close to that expected from mid-ocean ridge basalts (MORBs), indicating that it is the mantle wedge above the slab, rather than the slab itself, that melts. The significant exception to this observation is at the Banda arc, where the ratios are much lower, indicating contamination from the subducting Australian continent. It is the data from the Sunda and Banda arcs that Hilton and Craig² now examine more closely. The authors demonstrate that the change from higher (MORB) ratios to lower (contaminated) values occurs over a narrow (200 km) zone. This transition corresponds with a well-defined zone of seismicity, which they interpret as faulting between the subducting Indian ocean and Australian continental lithospheres⁴ (Fig. 2).

Gross differences in gravity, morphology, seismicity and volcanism between the Sunda and Banda arcs have long been known⁵. These differences have been best

explained by the change in subduction from oceanic to continental crust. Hypothetical transform faults have been proposed to explain some surface geological variations, but these have not been substantiated by close scrutiny. The seismological studies¹ were the first to indicate a narrow zone of fault activity within the lower plate.

Hilton and Craig find that R/R_a ratios in the eastern Sunda arc, which includes Java, Bali, Sumbawa and western Flores, exceed those on eastern Flores and Pantar by factors of 2–4. Passing east of a

rocks themselves.

But why the sharp transition? If the contamination is by sediments, would not the effect be gradual? The answer may lie in the structure of the western edge of the Australian continent. South-east of Sumba island, the lower plate has a fracture zone (an ancient transform fault) that divides the trench over a very narrow zone, about 25 km wide⁶. The fracture zone trends north-east, and its extension would place it very near the transition zone mapped geochemically by Hilton and Craig. East of the fracture zone, sediments several kilometres thick are entering the trench and a wide accretionary wedge is formed by scraping off the subducting trench sediments and accreting them onto the fore arc. West of the frac-

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FIG. 1 Evidence of activity: Banda volcano on the eastern section of the Sunda – Banda arc.

zone 500 km wide without active volcanism, three volcanoes of the Banda arc have values 4–8 times lower than those of the Sunda arc. The transition from high (MORB) values to low values occurs over a zone just 200 km wide. The zone of high seismicity within the lower plate occurs at the eastern end of this geochemical transition.

It is reasonable to conclude from these studies that it is oceanic lithosphere that underlies the Sunda arc west of central Flores, and that contamination of the mantle wedge begins beneath eastern Flores. This initial zone of contamination is west of the zone of intense seismicity in the lower plate, so the latter may represent the transition from oceanic to continental lithosphere. The contaminants seen geochemically by Hilton and Craig could then indicate melting of continental margin sedimentary rocks, rather than the crustal

ture the trench is nearly empty and the trench slope is steep, suggesting little accretion. If the surface structure shows so sharp a transition, might not the deeper mantle wedge be similarly affected? The results of Hilton and Craig imply that it is. They also show an anti-thetic relation between $^87\text{Sr}/^{86}\text{Sr}$ and R/R_a for the Sunda arc, but not for the Banda arc. This relationship supports the idea that two-component mixing of He and Sr is occurring over a large segment of the plate boundary, and has not been previously documented.

One might ask whether the Sunda-Banda arc structure is unique, or are there other examples? Poreda and Craig³ measured somewhat lower values of R/R_a for Taiwan, a well-documented zone of subduction of continental crust. The ratio is slightly reduced in the eastern Aleutians, the Kuriles and Kamchatka, and Japan, which may be explained in part by thick sediment input to these trenches. No reduction is seen, however, in the Cascades arc, whose trench is thickly sedimented. No He data are yet available

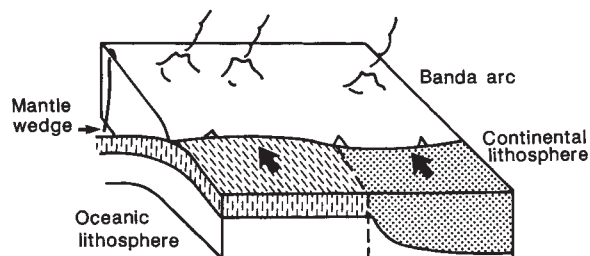


FIG. 2 Schematic diagram of transition between oceanic and continental lithosphere in Australian plate subducting beneath the Banda arc. Note source of arc magma in mantle wedge, generally about 100 km below the surface.

for the New Britain arc and the volcanoes north of Papua New Guinea, which lies in a somewhat different collisional regime to the Banda arc. The oceanic lithosphere

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beneath the Banda arc is continuous with the continent, and drags the continental rocks with it⁴, whereas in the Papua New Guinea collision the oceanic lithosphere appears structurally separated from the continent⁷. That difference may explain the unique results of Hilton and Craig in the Banda arc — and it can be tested.

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CELL BIOLOGY

Mitosis and membranes

Graham Warren

WHEN cells divide, the chromosomes segregate and all other cellular components are partitioned between the daughter cells¹. For most membrane compartments in animal cells the mechanism involves wholesale break-up; the cell, in effect, homogenizes the membranes yet does so in a manner which permits reassembly of the organelles. The pieces disperse randomly throughout the mitotic cell cytoplasm so that partitioning becomes a stochastic process dependent on the ability of the mother cell to divide itself into two daughters of equal size². The molecular mechanism whereby membrane compartments are broken down is obscure, but the report by Tuomikoski *et al.* on page 942 of this issue³ may provide a clue to what happens.

Morphological studies on organelle division go back to the beginning of this century⁴, but progress was slow until the advent of electron microscopy and, more recently, immuno-electron microscopy. There are only single copies of the endoplasmic reticulum and Golgi apparatus in animal cells^{5,6}, so they have to be divided during mitosis. They are first broken into fragments and then vesiculated. The nuclear envelope always breaks down in animal cells, but the extent of vesiculation of the rest of the endoplasmic reticulum varies from cell to cell⁷, perhaps because this organelle is present throughout the cell cytoplasm, and so does not need to be dispersed in order for partitioning to occur. In contrast, the Golgi apparatus, which has a restricted, juxtanuclear location⁸, is completely vesiculated to ensure its dispersal and consequent partitioning⁸.

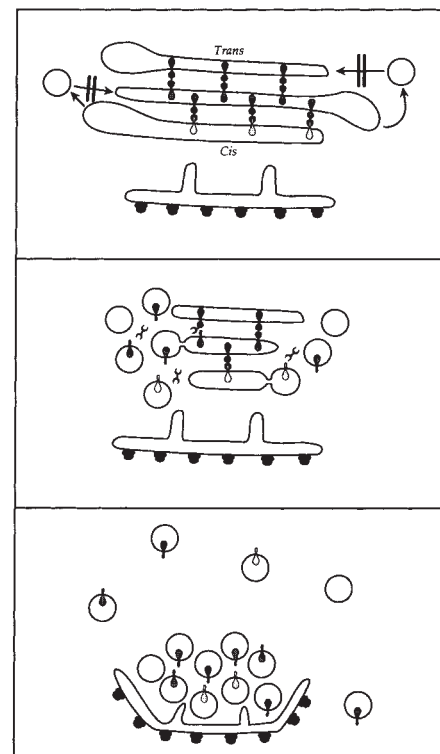
Despite the apparent complexity of this process it is possible to suggest a very simple model for vesiculation⁹ (see figure). The model demands separation of Golgi cisternae and cessation of vesicle fusion. Continued budding then converts the cisternae into free vesicles and clusters of vesicles which have been observed by immuno-electron microscopy^{10,11}. Many

lines of evidence point to an inhibition of membrane fusion during mitosis^{9,12}, but the evidence has, up to now, been indirect.

Tuomikoski *et al.*³ have now shown that membrane fusion is inhibited in extracts from mitotic cells. Isolated endocytic vesicles were mixed in the presence of an energy source and frog cytosol, and fusion was shown to occur. When the cytosol was prepared from frog eggs arrested in metaphase II, fusion was inhibited. They also treated interphase cytosol with the sea urchin homologue of p34^{cdc2}, the protein kinase of *cdc2*, which plays a key role in driving cells into the mitotic state¹³. This treated cytosol prevented fusion in a dose-dependent manner. Tuomikoski *et al.* have not, so far, shown that the effect of the extracts is reversible, but the absolute dependence of the reaction on p34^{cdc2} is evidence in favour of their interpretation.

The link between endocytosis and intra-Golgi transport is provided by an *N*-ethylmaleimide-sensitive factor (NSF). This was known to be involved in the fusion of Golgi transport vesicles¹⁴ and recent work has shown it to be involved in the fusion of endocytic vesicles¹⁵. Either NSF, or an associated protein of the fusion machinery, could therefore be the target for the inhibition during mitosis. But why inhibit the endocytic pathway? Is it simply the inevitable consequence of sharing the same fusion mechanism with an organelle that has to partition during mitosis? Or is it that endosomes are more elaborate and extensive than has so far been appreciated¹⁶, and that they too have to be vesiculated and partitioned in the same way as the Golgi apparatus? It could be that most, if not all, intracellular compartments that communicate by way of vesicles are present in only one or a few copies and inhibition of membrane fusion provides the means of partitioning during mitosis.

The reason for having a single copy is not known, but it may be a device to unify



Model for the vesiculation of the Golgi apparatus. Transported proteins move through the Golgi stack by way of vesicles which bud from the dilated rims and fuse with the next cisterna in the stack towards the *trans* side²⁰. Transport occurs by bulk flow²¹, which means that endogenous Golgi proteins such as the mannosidases and glycosyltransferases have to be prevented from entering the dilated rims. Lateral interactions might restrict their ability to act on glycoprotein substrates, so the simplest way to restrain them would be to link them to those proteins in the neighbouring cisterna which also need to be restrained. This would automatically generate the stack of cisternae which is the central feature of the Golgi apparatus. At the onset of mitosis transport vesicles would no longer be able to fuse (top panel) but budding would continue. At the same time, or shortly thereafter, the links between the cisternae would be broken allowing the endogenous Golgi proteins to move into the budding vesicles (middle panel). Continued budding would convert the cisternae into the vesicles (bottom panel) which are observed in metaphase animal cells¹⁰. Clusters of vesicles are also observed¹¹, though the material that binds them together has yet to be characterized.

the actions of an organelle. The resultant complex structures can then pose problems if the organelle needs to be moved to a new location in order to carry out a different function¹⁷. There is no reason why the partitioning mechanism should not work, during interphase, to facilitate such movement by way of vesicles. Mitosis would then represent the extreme when single-copy organelles are completely fragmented for partitioning.

Finally, one could ask why such a mechanism is used. Chromosomes, after all, use the mitotic spindle, so why is there not a more elaborate mechanism for

segregating membrane-bound organelles? There are several possible reasons. First, unlike the chromosomes, there is no need to divide an organelle into two exactly equivalent pieces. If one daughter re-

ceives slightly more or less membrane, then mechanisms exist to correct the imbalance¹⁸. Second, the mechanism is straightforward in that it inhibits a pre-existing process, though additional modifications may be needed to ensure that the vesicles contain the information needed for reassembly¹⁰. Third, it is general because most, if not all, vesicle-mediated pathways share the same mechanism for fusing membranes, specificity being provided by other proteins¹⁹. Last, it is flexible. The vesicles generated are small and easily diffusible, so they should rapidly and randomly occupy the mitotic cell cytoplasm. This means that it does not matter what shape or size the cell is, so long as the cytokinetic mechanism divides it into two daughters of equal size³. The molecular mechanism underlying this membrane-partitioning process is of obvious interest and the report by Tuomikoski *et al.* is a step in the right direction. □

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sion with which such translocation can be predicted, and on more detailed evidence concerning the stability of the carbon reservoir in the soil.

Long *et al.*² present new results from a continuing UNEP study of four tropical grassland sites in Mexico, Kenya, Thailand and Brazil. They used 20 randomized sample plots, each 1.0m by 0.25m in size, which they cropped every month and determined the weight of living and dead tissues, both above and below ground. The root material was extracted from the soil cores using fine-mesh (2mm) sieves (no real advantage was found in using sieves of smaller aperture). They separated the live roots by staining with tetrazolium salts.

They were then able to calculate the turnover of plant matter, both above and below the soil surface, that is neglected in most standard procedures. In three of their sites they found that the traditional IBP methods had underestimated primary productivity by a factor of between two and five. On a global scale this means that there is substantially more productivity in the tropical grassland biome than has previously been realized.

These results have a bearing on both agricultural and carbon-cycling studies. In pastoralism, they imply that more energy is entering the ecosystem than had been thought. If a substantial amount of leaf material is dying and passing to detritivores, then one must conclude that the grassland is being under-used. Energy diverted to roots, however, is of no economic value to grazing animals. But even this aspect of carbon allocation has implications for global carbon-budget studies, for clearly there is a larger sink for carbon in the tropical grasslands than has previously been suspected, but only if some of the added carbon is accumulating in the soil. This does seem to be occurring in some of the grassland sites examined by Long *et al.* but much more effort needs to be expended on precise quantification of this build-up of organic matter. Equally important, the ploughing of grasslands for arable agriculture will inevitably result in the release of soil carbon back into the atmosphere. Perhaps the ostrich, with its head below the ground, was actually trying to tell us something. □

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ECOLOGY

Answers that lie in the soil

Peter D. Moore

OUT of sight, out of mind, may be a convenient maxim for those with the inclinations of an ostrich. But it is hardly appropriate for ecologists concerned with estimating the productivity of the Earth's ecosystems, for much production occurs beneath the surface of the soil. The International Biological Programme (IBP) of the 1960s placed strong emphasis on the development of techniques for studying productivity and many data were accumulated which have subsequently been used in the assessment of the productive resources available for human support and, more recently, in developing a fuller picture of the Earth's carbon cycle. But many of these initial results are flawed by a failure to account adequately for the underground accumulation of carbon, and also for the continuous cycle of death and turnover of plant tissues above and below ground. Work by Raich and Nadelhoffer on forests¹ and by Long *et al.* on grasslands² now implies that some of the early results on this subject could be a serious under-estimate of the true values.

It would be wrong to suggest that the IBP pioneers were unaware of the problem of root productivity and turnover. Newbould³ proposed that the ratio of below-ground production to below-ground biomass is proportional to that above ground and Newman⁴ developed techniques for the measurement of the extent of plant roots within the soil by

dissecting out sample soil volumes. Many field studies, however, neglected the subterranean part of the equation simply because of the difficulties involved in its measurement. Others estimated changes beneath the ground by subtracting the minimum from the maximum root biomass in a given year (Dahlman and Kucera in their studies of prairies⁵, for example).

For forest ecosystems, there is considerable disagreement over the proportion of fixed carbon that is allocated to subterranean organs. Raich and Nadelhoffer¹ have collated and examined data derived from studies in a wide geographical range of forest ecosystems. They start by assuming steady-state conditions for their soil carbon cycle (which may itself be questionable) and derive an expression to show that the total carbon allocation to roots can be estimated by summing root detritus production and root respiration; this, in turn, is given by the soil respiration rate less the rate of above-ground litter production (which supplies soil heterotrophs with their additional source of carbon). Raich and Nadelhoffer conclude that there is a positive linear relationship between litter fall and carbon allocation to roots, but that, in proportional terms, relatively less of the fixed carbon is transported to roots in more productive forests. Ultimately, our knowledge of terrestrial carbon cycles will depend upon the preci-

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SYNTHETIC CHEMISTRY

Chemists get topological

Roland Pease

MOLECULAR LEGO is the term Fraser Stoddart and colleagues have coined to describe their approach to chemical synthesis. Yet this almost understates the ease with which the reagents assemble themselves to form the catenane — a linked-ring compound — depicted below (Fig. 1). Their new work¹ builds on both the host-guest chemistry for which Pederson, Cram and Lehn gained the Nobel prize, and the notion of topological isomers developed by Frisch and Wasserman².

Catenanes are characterized by the linking together of molecular rings without the intervention of covalent bonding. The importance of the new work is in the exploitation of non-covalent 'donor-acceptor' interactions that make the reagents act as templates for one another. In this way, the authors obtain a 70 per cent yield of the catenane product from a simple one-step reaction (stir reagents 2, 3 and 4 — Fig. 2 — together in a solvent and leave to stand overnight); and that yield represents merely the red precipitate deposited and does not take into account any product still in solution. By comparison, other synthetic routes are both more complex and less efficient.

The key to the authors' success was the recognition of the non-covalent forces at work in host-guest complexes. They noted that the roles of electron donor (red) and acceptor (blue) are interchangeable in binding the supramolecular complexes 5 and 6 (Fig. 3). This reciprocity, they argued, could be used to stabilize a reaction intermediate in which an electron-donor guest ring (compound 4) would hold, vice-like, one limb of the u-shaped acceptor compound 2. Effectively, the two rings of the catenane — one initially

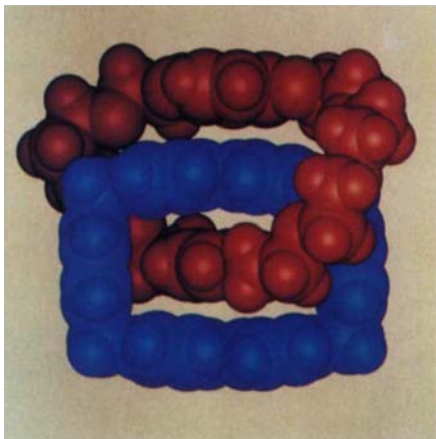


FIG. 1 Space-filling model of the [2] catenane (compound 1) synthesized by Stoddart and colleagues. Prepared on the basis of X-ray data by D.J. Williams and colleagues at Imperial College.

incomplete — act mutually as templates for the reaction (Fig. 2), which is completed by the ring closure by moiety 3. The ingenuity deployed in the new work, the culmination of ten years of systematic study, is characteristic of studies in 'topological' chemistry.

Previous attempts to synthesize catenanes have relied on the improbable threading of a large cyclic molecule onto a long single-strand molecule, on the

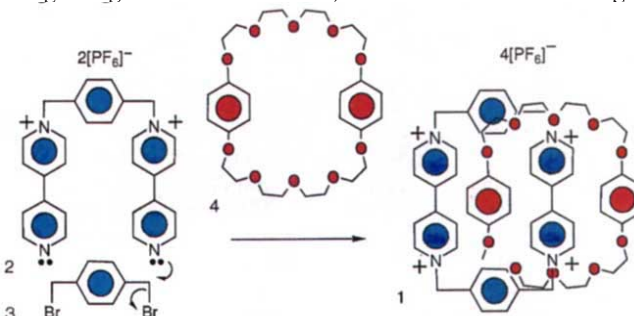


FIG. 2 Reaction scheme by which the [2] catenane (compound 1) is formed from a bispyridinium salt (2^{2+}), $2PF_6^{-}$, 1,4-bis(bromomethyl)benzene (3) and bisparaphenylene-34-crown-10 (4). The reaction was carried out at room temperature in dry acetonitrile (Blue, electron acceptor; red, electron donor.)

remarkable topological properties of the Möbius strip, and on the ability of transition-metal ions to arrange organic ligand molecules in fixed orientations. The first method was also the first to work³. The two reagents are mixed in conditions that favour closure reactions of the linear molecule. In the rare event that ring closure occurs after the linear molecule has threaded the macrocycle, the product is a catenane that is topologically distinct from the main reaction product. Although only traces of catenane were made, their presence could be demonstrated by subtle use of deuterated reagents and analytical chemistry.

Cut a Möbius strip with two twists, and the result is a pair of rings with the same radius, but inextricably linked. The notion lends itself well to catenane synthesis, but the execution is not trivial. The problem is to find a double-stranded molecule with breakable crosslinks that is sufficiently flexible both to turn back on itself for ring closure, to form a loop and to twist twice on its axis. Nevertheless, a Möbius strip was successfully made (22 per cent yield) with a single twist in 1982 by D.M. Walba and colleagues⁶. The earlier attempt by Wasserman and colleagues^{5,6} to synthesize the double-twist Möbius strip led only to trace quantities of catenane.

By far the most successful method to date is the use of transition-metal ions as templates for catenane synthesis. This approach, espoused by J.-P. Sauvage and colleagues⁷, relies on the directionality of

transition-metal-ligand bonds to dispose rigid reagent molecules in the correct arrangement for catenane synthesis. The ligands are either two half-rings or a half and a whole-ring which overlap and are completed by the addition of complementary half-rings. The final step in this synthetic route is to extract the strongly bound metal ion from the middle of the newly formed catenane without undoing the product. It turns out that the powerful ligand cyanide (CN^{-}) works admirably and yields of 50 per cent with respect to the starting materials are common. Not only have Sauvage and colleagues succeeded in making [2] catenanes (two interlocking rings) by this method, but also [3] catenanes and a trefoil knot — the simplest topological knot of a single strand.

The significance of the new work by Stoddart and colleagues is not restricted to the synthesis of an unusual molecule. The product is constructed from components that enter into charge-transfer interactions, the basis of conducting (and super-

conducting) organic crystals, and so has interesting electrical properties. Most notably, electrochemical experiments show that the reduction of the quadruply charged, positive molecule (its acceptance of electrons) varies with the environment of the accepting units relative to the donor, the reduction potential depending on whether they are on the inside or outside of the donor macrocycle. Nuclear magnetic resonance experiments show that the molecule has two 'internal melting' points — a lower one at which the acceptor part (with the bipyridinium units, blue) starts to 'pirouette' around the axis defined by the interior $O-C_6H_4-O$ unit of the donor ring, and a higher one at which both rings become free to slip around one another.

Most of all, however, the work

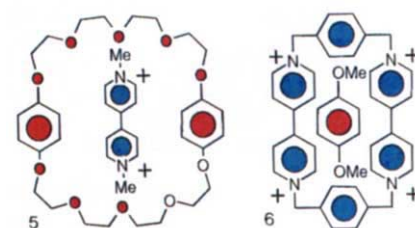


FIG. 3 Host-guest complexes of the paraquat di-cation (blue) with bisparaphenylene-34-crown-10 (red) (compound 5) and of cyclobis(paraquat-p-phenylene) (blue) with 1,4-dimethoxybenzene (red) (compound 6) which were the models for the donor-acceptor forces stabilizing the intermediate in the synthesis of the [2] catenane (compound 1).

demonstrates the power of charge-transfer forces in allowing molecules to form their own template for chemical

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reactions. This effect lies at the very heart of the structure-directed methods of Stoddart and colleagues. If anything, Stoddart suggests there may even be an element of self-catalysis in the reaction — at least this is what seems to be indicated by the kinetics of the reaction. The authors are now working on a [3] catenane which, by all accounts, is easier to synthesize than its constituent parts. □

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MAMMALIAN SEX DETERMINATION

Thumbs down for zinc finger?

Paul S. Burgoyne

TOWARDS the end of 1987 *Cell* published a paper by Page *et al.*¹ which convinced many (myself included) that the mammalian sex-determining gene, which directs the developing fetal gonad to form a testis, had been identified on the human Y chromosome. The gene in question encoded a 'zinc finger' protein (such proteins are believed to interact directly with DNA) and has been dubbed *ZFY* (zinc-finger Y); intriguingly, a very closely related gene, *ZFX*, was found on the X chromosome. I suspect I was not alone among those involved in trying to unravel the process of mammalian sex determination, who, on hearing this news, considered taking early retirement. Yet the months slipped by without the expected reports of *ZFY* expression in the developing fetal testis, although the equivalent mouse sequences were shown to be expressed in adult testes^{2,3}. There then appeared a paper by Sinclair *et al.*⁴ which revealed that *ZFY*-related sequences in marsupials were not on the Y chromosome. While some took refuge in the heresy that the Y chromosome might not be male-determining in marsupials, I abandoned for the moment thoughts of retirement to a cottage in the country. Now, two years after the discovery of *ZFY*, two papers in this issue of *Nature*^{5,6} appear to exclude *ZFY*, and the mouse equivalent *Zfy*, from having a testis-determining role. (By convention, human gene labels are all capitals, whereas mouse gene labels have only the first letter as a capital. When referring to equivalent genes without respect to species I will use capitals in inverted commas — '*ZFY*', for example.)

Koopman *et al.*⁵ (page 940) provide the long-awaited description of '*ZFY*' expression in the developing fetal testis. In the mouse there are two Y-chromosomal homologues of '*ZFY*', which are designated *Zfy-1* and *Zfy-2*; but because it had previously been shown that testes develop in the absence of *Zfy-2* (refs 3, 7), interest centred on *Zfy-1*. The initial results obtained by Koopman *et al.*

seemed to strengthen the case that *Zfy-1* is the primary testis determinant: the first *Zfy-1* transcripts appear just before testis differentiation commences, they increase in abundance as testis differentiation ensues, and then decrease once testicular differentiation is established. It is to the authors' credit that they did not rush into print at this point. Instead, bearing in mind that *Zfy-1* (and *Zfy-2*) expression in adult testes appeared to be germ-cell dependent⁵, they decided to see if *Zfy-1* expression occurred in fetal testes lacking germ cells. The answer was clear — no germ cells, no *Zfy-1* expression.

Now the pattern of *Zfy-1* expression can be viewed in a very different light: the appearance and increase in *Zfy-1* transcripts is correlated with the colonization of the developing gonad with germ cells, while the subsequent decrease in *Zfy-1* expression correlates with the cessation of germ-cell proliferation and dilution of these cells with the expanding somatic components of the testis. It remains to be seen how far back in the germ-cell lineage *Zfy-1* expression occurs. Because testis determination is mediated by Y-chromosome expression in the Sertoli cell lineage⁸, and can occur in the virtual absence of germ cells, one must accept the authors' conclusion that their observations "exclude both *Zfy-1* and *Zfy-2* as candidates for the mouse testis-determining gene".

What of '*ZFY*' in man? Since the paper by Page *et al.* appeared it has become clear that some XX men and perhaps all XX true hermaphrodites (that is, individuals with ovarian and testicular tissue) lack *ZFY*, and that, in some instances, *ZFY*-negative XX males and hermaphrodites can occur in the same families. For reasons I shall come to later, I became convinced that these familial XX males and hermaphrodites could best be explained if the primary testis-determining gene was distinct from *ZFY* and lay very close to the pseudoautosomal boundary. I shall refer to this primary testis-determining gene as *TDY* (*Tdy* in

the mouse). If the original 'mutation' giving rise to these familial cases of XX sex-reversal involved an X–Y interchange, then they should be positive for Y-specific sequences adjacent to the pseudoautosomal boundary.

Palmer *et al.*⁶ (page 937) have examined 14 males or hermaphrodites who lacked *ZFY*; three of the males and one of the hermaphrodites did indeed carry Y-specific pseudoautosomal boundary sequences. Although the remainder lacked the boundary, they could have had small insertions that include *TDY*, rather than having mutations "elsewhere in the testis-determination pathway", as Palmer *et al.* suggest. The authors conclude that "these results lead to the strong prediction that a gene involved in sex determination lies close to or even spans the pseudoautosomal boundary". The hunt is now on!

So, do we now conclude that Page *et al.*¹ were wrong and leave it at that? I think not, because it would leave too many loose ends. Palmer *et al.* suggest that the only discrepancy between their findings and Page's lies in the latter's conclusion that at least part of the testis-determining gene must fall within the region of the Y chromosome missing in 'XY' female WHT1013. This 'XY' female in fact carried a reciprocal translocation between the Y chromosome and chromosome 22 (Fig. 1). Presumably loss of the region of the Y chromosome that includes *ZFY* occurred in conjunction with this reciprocal exchange. The crucial question is whether this individual is female because of the deletion of *ZFY*, or because of some other effect of the translocation. Palmer *et al.* favour the second explanation; they suggest that the testis-determining gene (located near the pseudoautosomal boundary) could have been inactivated through a 'position effect' because this

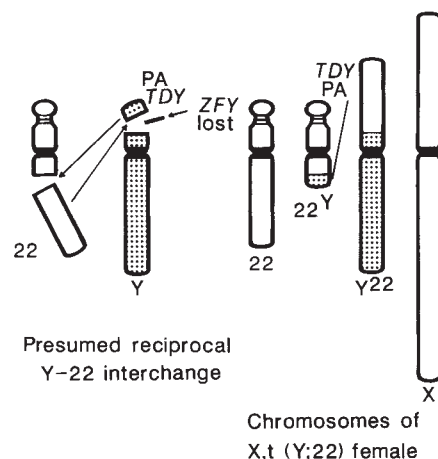
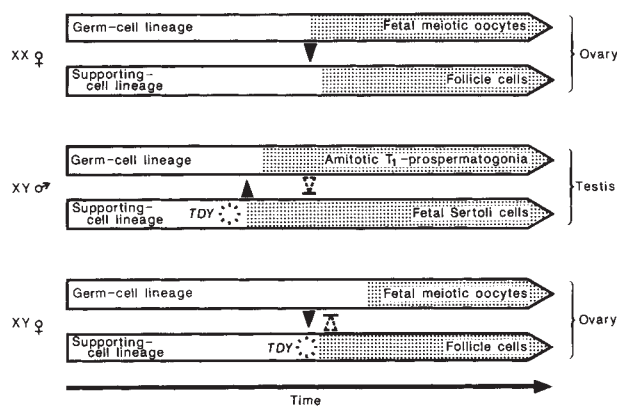


FIG. 1 The deduced arrangement of Y-chromosomal material (shaded) in the 'XY' [46,X,t(Y;22)] female WHT1013 of Page *et al.* (see ref. 1). Note that the Y pseudoautosomal region and, according to Palmer *et al.*⁶, the testis-determining gene (*TDY*), are on the 22^Y chromosome. *ZFY* has been lost during the exchange.

FIG. 2 A model for mammalian sex-determination and XY sex-reversal. In the 'default' pathway to ovarian development (top panel), the germ-cell lineage, as a normal consequence of its differentiation, produces a diffusible ovary determinant which commits the somatic supporting-cell lineage to form follicle cells. In the diagram the production of the ovary determinant is arbitrarily shown to occur around the time the germ cells enter meiosis. In the male (second panel) *TDY* directs the somatic supporting-cell lineage to form fetal Sertoli cells. It is envisaged that these cells retain the capacity to respond to the ovary determinant. However, the fetal Sertoli cells carry out a 'pre-emptive strike' against the germ cells, committing them to the male pathway as T_1 -prospermatogonia, thereby blocking the production of the ovary determinant. In the text it is suggested that one route to XY sex-reversal is via a developmental delay in the gonadal soma, so that the germ-cell lineage produces the ovary determinant *before* the pre-emptive strike from the supporting-cell lineage can occur (lower panel). The involvement of altered growth rates in sex-reversal has been argued by Mittwoch¹⁸, although with a different mechanism to that illustrated here.



part of the Y is now attached to chromosome 22. In what follows I will try to argue a case for the alternative possibility: that deletion of *ZFY* was, as Page *et al.* supposed, the cause of the sex reversal.

In arguing the case for a role for '*ZFY*' in sex determination in man (but not in the mouse), my starting point is the striking difference between the two species in '*ZF*' gene expression in somatic tissues. In humans *ZFX* and *ZFY* are ubiquitously expressed, and *ZFX* is not dosage-compensated by X-chromosome inactivation¹⁰. Thus the normal expressed '*ZF*' gene dose is two — (2 *ZFX*, or 1 *ZFX* + 1 *ZFY*). In mice, only *Zfx* is expressed in somatic tissues, and it is almost certainly dosage-compensated by X-chromosome inactivation, so that XX cells have one active copy of the gene (R. Lovell-Badge, personal communication). This dosage compensation makes sense because it equalizes the '*ZF*' dosage in males and females, the normal expressed '*ZF*' dose in mice being 1 *Zfx*. Consider now that mouse XO individuals show only a mild retardation early in embryonic life¹¹, and have normal prenatal and postnatal viability. In marked contrast, human XO individuals are so seriously affected that more than 95 per cent of those conceived do not survive to term, and those that do are subject to the range of defects which constitute Turner's syndrome.

It does not require a major leap of imagination to attribute the severe developmental abnormalities in human XO individuals to their deficiency in '*ZF*' gene dosage, and to attribute the anti-Turner effect of the Y (men have one X chromosome but do not have Turner's syndrome) to the observed expression of *ZFY* in somatic tissues. Indeed, this anti-Turner effect of the Y maps to the same

region as *ZFY*¹². In mice, where the norm is one expressed dose of *Zfx*, XO individuals will only be at a disadvantage in the early embryonic period, when, before X-inactivation, XX embryos could be expressing two doses of *Zfx*.

I now want to introduce a concept relating to XY sex-reversal which will be crucial in the arguments that follow. The obvious route to XY sex-reversal is through loss or inactivation of the testis-determining gene '*TDY*', and there is tantalizing mention of just such a mouse mutant at the end of the paper by Koopman *et al.*⁵. But an alternative route, for which there is convincing evidence in the mouse^{13,14}, is one where there is a 'timing mismatch' between the testis-determination process and the ovary-determination process, such that '*TDY*' fails to pre-empt ovarian development. My own working hypothesis for how this might operate is explained in Fig. 2. This hypothesis predicts that if the development of the XY gonadal soma is slowed relative to the germinal component, then an ovary can develop. Although '*TDY*' is present it 'misses the boat'.

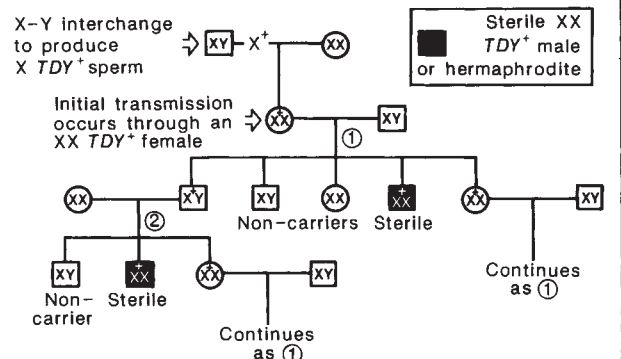
There are some human pedigrees in which the pattern of inheritance of XY sex-reversal clearly implicates an X-linked mutation. Paradoxically, in the mouse where the extensive breeding of X-chromosome-marked stocks should easily detect such a mutation, no X-linked cases of XY sex-reversal have been identified. A resolution to this paradox can be provided by bringing together the arguments presented in the preceding two paragraphs. If a double-expressed '*ZF*' dose is needed for normal human somatic development, and retardation of the somatic compartment of an XY fetal gonad can lead to sex-reversal, then a mutation that interfered with human *ZFX* expression could result in XY sex-reversal by the 'timing mismatch' route. In the mouse, where a single expressed *Zfx* dose is the norm, interference with *Zfx* expression would be expected to be lethal in early embryogenesis, thus precluding this as a route to XY sex-reversal.

It is now only a small step to explaining the sex-reversal of Page's *ZFY*-negative 'XY' female WHT1013. In man, *ZFY* contributes to the expressed '*ZF*' dose in somatic tissues, so deletion of *ZFY* could result in retardation of the gonadal soma, and thus once again lead to XY sex-reversal by the 'timing mismatch' route.

Having concluded that a double '*ZF*' dose is necessary in man to ensure that *TDY* can pre-empt the ovarian pathway, we can now return to the familial cases of *ZFY*-negative XX males and hermaphrodites. I would argue that testicular tissue develops in these individuals because they not only have *TDY* (located close to the pseudoautosomal boundary) but they also have a double '*ZF*' dose (2 *ZFX*). These familial cases raise two further questions, however: how can the trait be transmitted when XX males are always sterile, and why are there so many hermaphrodites?

A lesson we have learned from mice is that hermaphroditism can only be achieved by walking a developmental tightrope: the system is strongly canalized to give either ovaries or testes. Indeed, hermaphrodites frequently have an ovary on one side and a testis on the other, rather than ovotestes. Where a particular

FIG. 3 Proposed scheme for the inheritance of *TDY*-positive, *ZFY*-negative XX sex-reversal. It is suggested (see text) that XX, *TDY*⁺ individuals can develop not only as sterile males and hermaphrodites, but also occasionally as fertile females, the latter holding the key to the transmission of the trait.



genotype does produce some hermaphrodites, genotypically identical males and females are also produced. Thus I would argue that familial transmission of XX sex-reversal occurs in the first instance through a *TDY*-positive female. In the next generation transmission is possible through XY male carriers (Fig. 3). But if this hypothesis is correct, we have to explain not only how you can get *TDY*-positive hermaphrodites, but also how *TDY*-positive females arise.

I suppose that by analogy with T16/*Xsrx* mice, which can develop as females, males and hermaphrodites^{15,16}, a spreading of X-inactivation into *TDY* could be invoked as the cause of the variation in sexual phenotype (in these mice, the X chromosome bearing the sex-reversing factor *Sxr* is held in the inactive condition by a translocation between the X chromosome and chromosome 16). The fact that *ZFY*-positive sex-reversed XX individuals are invariably male would then have to be attributed to the interposition of more Y chromosomal material between *TDY* and the X chromosome in these cases. An alternative explanation for the occurrence of *TDY*-positive females, which I find attractive, brings us back once more to *ZFY*. Although I have argued that *ZFY* and *ZFX* are functionally interchangeable in the soma, the proteins they encode are not quite identical. It is therefore possible that *ZFY* could exert a stronger effect than *ZFX*. If so, 1 *ZFX* + 1 *ZFY* could be necessary to ensure that *TDY* pre-empts the ovarian pathway, 2 *ZFX* representing a borderline dose in this respect so that *TDY* sometimes 'misses the boat'. The *ZFY*-positive XX individuals should always be male because, in addition to *TDY*, they have overkill in terms of '*ZF*' dosage (2 *ZFX* + 1 *ZFY*).

So far we have only viewed '*ZFY*' as a '*ZFX*' substitute. What then is the evidence for '*ZFY*' having any truly male-specific role? The high levels of '*ZFY*' expression in adult testes are certainly a strong indication of a male-specific function, and I have suggested elsewhere¹⁷ that

Zfy (principally *Zfy-2*) could be the mouse spermatogenesis gene *Spv*; *Spv* is required from about one week after birth for the normal proliferation and survival of spermatogonia. The evidence that *Zfy-1* is expressed in the germ cells of fetal XY mouse gonads does not necessarily imply a male-specific role for the Y in the germ line at this earlier stage. XO germ cells in XO/XY mosaics, despite lacking *Zfy-1* and *Zfy-2*, certainly form fetal prospermatogonia, and go on to form definitive spermatogonia post-natally. Perhaps *Zfy-1* in the fetal XY gonad is once again just standing in for *Zfx*. After all, both X chromosomes become active in the germ line of females around this time.

The results now published by Koopman *et al.*⁵ and Palmer *et al.*⁶ apparently exclude '*ZFY*' from being the testis-

determining gene. I have argued that *ZFY* in man, could, together with *ZFX*, be necessary to ensure that the testis-determining gene can pre-empt ovarian development. If this is correct, then the human Y-chromosomal 'testis-determining factor' *TDF* can be equated with *TDY* + *ZFY*.

If the views presented here prove to have any validity, then many people who have shared their ideas with me deserve credit. I would especially acknowledge the contributions of Robin Lovell-Badge and Jean Weissenbach. If this all proves to be nonsense, there is always that cottage in the country. □

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VOLCANO PREDICTION

Measures of little gravity

Robert I. Tilling

THE measurement and mapping of anomalies in the Earth's gravity field constitute a powerful geophysical technique for studying crustal structure. The detection of minute changes in the gravity field preceding volcanic eruptions is an aspect of especial interest. The causal link between changes and eruption is often hard to identify unambiguously, but Rymer and Brown, reporting on page 902 of this issue¹, suggest that a mechanism is discernible for microgravity changes preceding the eruption last spring at Poás Volcano in Costa Rica. The variations reflect, they argue, the shallow intrusion of magma beneath the volcano's summit crater lake and its subsequent cooling and vesiculation, and the evaporation of lake water. More importantly, the authors imply that the trend of gravity increase that started in 1985 was a precursor of the explosive eruption in April–May this year.

Although gravity measurements were attempted in the late 1930s at Mont Pelée (Martinique, Lesser Antilles) in an effort to identify a precursory phenomenon, it was not until the development of high-precision gravity meters in the 1950s that successful studies of temporal variations could be made². With modern high-sensitivity instruments and well-designed field-measurement techniques, gravity changes at a volcano can be measured to a precision of 10^{-5} (10 microgal) or better^{3–6}. After correction for Earth tides and instrumental factors, measurable variations in gravity can be attributed to displacements of the observation point resulting from deformation produced by the intrusion or withdrawal of subsurface magma and the redistribution of mass in the volcanic plumbing system.

Fortunately, the free-air effect of eleva-

tion change on the observed gravity can be calculated if the elevation is also monitored. Alternatively, it can be estimated indirectly by periodic reoccupation of geodetic networks deployed to monitor ground deformation at volcanoes. Once the elevation changes are allowed for, the gravity residuals, if any, can be interpreted in terms of various models of mass redistribution affecting the volcanic system^{2,3,7}: surface and subsurface movement of magma, particularly the filling and draining of a near-surface magma reservoir; changes in density of the magma bodies, through degassing, vesiculation (cavitation), cooling and contraction, or hydrothermal alteration; and changes in the surface and subsurface hydrological and geothermal regimes.

Poás is one of several active volcanoes in Costa Rica. Virtually all of its historical eruptions have occurred at a highly acid, hot crater lake that lies at its summit. Accordingly, the activity of the volcano — the boiling of lake water and its eruption in geysers, the ejection of mud plumes, explosive, steam-driven (or phreatic) eruptions, and (less commonly) the ejection of pyroclastic sulphur and lava eruptions — reflects the complex interaction, at relatively shallow depth, between the crater lake, the subsurface hydrothermal system, and the magma body⁸. In recent decades, several Costa Rican research groups, in cooperation with earth scientists from elsewhere, have initiated or augmented seismic, geodetic and other studies at Poás. Rymer and Brown's measurements of gravity changes since 1979 contribute to the growing body of knowledge about the behaviour of Poás.

The gravity changes in the period 1979–85 (less than 120 microgal) show no

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particular trend, permitting the somewhat speculative interpretation that the variations result largely from 'cyclical' changes in density of the underlying magma related to degree of vesiculation^{1,7}. Since 1985, there has been a trend of increasing gravity (by up to 400 microgal) at some of the gravity stations, which Rymer and Brown attribute to a shallow magma intrusion which perturbed the hydrothermal activity of the crater lake. Ultimately, they suggest, this culminated in the vigorous phreatic explosions of April–May 1989. This interpretation, and the supporting data for it, are clearly summarized in figures 1–3 of their paper¹.

Unfortunately, as Rymer and Brown acknowledge, the elevation monitoring at Poás Volcano was poor before 1987. The authors argue that elevation changes before 1987 were probably not significant. But without the data, and the consequent allowances in interpreting the gravity data, their model, although certainly plausible, cannot be substantiated.

The elevation controls since 1987 need to be considered in the context of other monitoring data from Poás, one of the best-observed volcanoes in Latin America. In addition to continuous seismic monitoring, various geodetic (electronic tiltmeter, precise-level line, and ground-tilt arrays), thermal and geochemical measurements are made periodically at Poás⁹. But such data largely remain unpublished. The limited data that have been reported through the Scientific Event Alert Network (Smithsonian Institution, Washington DC) suggest that measurable changes took place in the state of the volcano leading to the April–May 1989 explosions. For example the summit was sharply inflated during June–August 1988, deflation following in the next five months; seismicity increased by an order of magnitude after January 1989; and there was a shift in the site of vigorous thermal activity in March 1989, suggesting¹⁰ that "magma is rising below the lake . . . could lead to an eruptive event". These observations need to be incorporated with Rymer and Brown's to strengthen or modify their interpretation.

Can microgravity be used for predicting volcanic eruptions? For now, the strict answer has to be "no": a 'prediction' is generally defined¹¹ as "a comparatively precise statement of the time, place, and

ideally, the nature and size of impending activity" made before the activity occurs. Although the microgravity studies at Poás and other well-studied volcanoes clearly show that the method is capable of detecting and monitoring magma movement, they do not yet meet the full rigour of this definition. I would fully concur with Rymer and Brown¹ that microgravity surveys have "considerable potential for understanding magma chamber dynamics and eruptive precursors, especially when [my italics] combined with accurate height control", if the words *only if* were substituted for *especially when*.

Lastly, microgravity surveys require the

operator to be on site — not necessarily a felicitous situation for the operator given the explosive nature of the activity at many volcanoes. Techniques suited to remote and continuous monitoring seem preferable for recording precursory processes immediately before an eruption. Nonetheless, experience indicates that volcano monitoring is best achieved by integrating many approaches, among which microgravity measurements should definitely be included. □

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IMMUNOLOGY

Making antigen-receptor genes

Gary Rathbun, Frederick W. Alt and George D. Yancopoulos

In a paper that appears on page 934 of this issue¹, and in a report in *Cell*², two groups describe the cloning of genes that encode proteins that may play critical roles in the assembly of antigen-receptor variable-region genes. The enormous diversity of antigen-binding specificities expressed by B and T cells of the immune system depends upon a strictly regulated, site-specific genomic recombination process. This recombination process assembles component gene segments (*V* for variable, *D* for diversity and *J* for joining) into variable-region genes that encode the antigen-binding portions of the various B- and T-cell antigen receptors (Ig and TCR, respectively). *V-(D)-J* recombinase activity has been assayed in cells through the use of introduced recombination substrates (containing unrearranged *V*, *D* or *J* segments flanking selectable marker genes). Such experiments showed that a single, shared *V-(D)-J* recombinase activity rearranges all three Ig and the four known TCR antigen-receptor variable-region gene loci; the recombinase is targeted to the respective gene coding segments by similar recombination recognition sequences (RS) that flank all Ig and TCR *V*, *D* and *J* coding segments. In addition, recombinase activity was generally found only in the early stages of B- and T-cell development and not in non-lymphoid cells (for review see refs 3 and 4). Based on this information, several strategies have been employed in the attempt to isolate the genes and/or the protein products involved in *V-(D)-J* recombination. These have included isolation of genes specifically expressed in early B- and T-lineage cells, isolation of genes encoding RS sequence-binding proteins, and isolation of genes that confer a recombinogenic phenotype on non-lymphoid cells.

Recombination recognition sequences consist of a palindromic heptanucleotide

sequence and an A/T-rich nonanucleotide sequence separated by a spacer of either 12 or 23 base pairs. *V-(D)-J* recombinase activity apparently only efficiently recognizes and joins a gene segment flanked by an RS with a 12-base pair spacer to a segment flanked by an RS with a 23-base pair spacer (12/23 rule). Complete understanding of the precise mechanism of *V-(D)-J* joining awaits the definition of the enzymatic machinery that carries out the process, although general aspects of this mechanism have been elucidated^{3,4}. The various activities required include recognition of the RS sequences, site-specific endonucleolytic cleavage between these RS sequences and the adjacent gene segments, potential nucleotide addition and removal activities at the ends of the severed segments (which increases coding diversity at the junctions between the segments), and ligation of the gene segments involved³. Apart from the enzyme terminal deoxynucleotidyl transferase, thought to be involved in adding extra nucleotides during junction formation and not to be essential for *V-(D)-J* joining, none of the component(s) involved in *V-(D)-J* recombination has been isolated.

Several groups have previously reported proteins that bind specifically to the heptamer or nonamer of the RS sequences^{5,6}. Matsunami *et al.*¹ now describe the purification from nuclear extracts of a precursor B-cell line of a protein of relative molecular mass 60,000 (60k) that binds to an RS, and the isolation of a complementary cDNA clone (*RBP-2*) based on the partial amino-acid sequence of this protein. The protein encoded by this cDNA does not have extensive overall homology with any known proteins, but displays an intriguing homology to a 40-residue region that is conserved among a subset of site-specific recombinase (the integrase family) in

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prokaryotes and the Flp recombinase in yeast. The authors suggest that this 40-residue region may represent the catalytic domain of *V-(D)-J* recombinase, with other regions of the proteins conferring binding specificity. But elucidation of the relationship of *RBP-2* to *V-(D)-J* recombinase activity awaits further information (see later).

Schatz, Oettinger and Baltimore² have isolated a 'recombination activating gene' (*RAG-1*) through its ability to activate *V-(D)-J* recombination when introduced into NIH3T3 fibroblasts⁷. The fibroblasts contained a recombination substrate that conferred drug resistance only when specifically rearranged by *V-(D)-J* recombinase; oligonucleotide-tagging of transfected genomic DNA fragments allowed for the isolation of the activating gene. Significantly, expression of *RAG-1* precisely correlates with expected (or measured) early lymphoid specificity of *V-(D)-J* recombinase activity in cell lines and normal tissues. The *RAG-1* gene encodes a 119k protein that is highly conserved between species. The only suggestive homology of *RAG-1* with known proteins was to the DNA-binding domain of the glucocorticoid receptor. Although these authors favour the idea that *RAG-1* encodes all or part of the *V-(D)-J* recombinase, they do not rule out the equally interesting possibility that *RAG-1* acts as a regulatory molecule that activates the *V-(D)-J* recombinase gene or protein.

Fibroblasts that express a transfected *RAG-1* gene do not express any of several other lymphoid-specific molecules, suggesting that either *RAG-1* is a very specific regulator of *V-(D)-J* recombinase or that it is a direct participant in the actual recombination process. In the second case, *RAG-1* would either act alone or recruit more ubiquitous accessory factors present at least in the NIH3T3 cell line used; there is a precedent for this, in that accessory factors are known to exist in prokaryotic systems⁸. Various analyses have indicated that the severe combined immunodeficient (SCID) mouse has a defect that impairs the final stages of *V-(D)-J* recombination^{3,4,9}. The SCID mutation maps to chromosome 16, whereas *RAG-1* apparently maps to a different chromosome. Thus the SCID mutation may affect a gene normally activated by *RAG-1* (if it is an activator) or a gene encoding an accessory factor recruited by *RAG-1* (if *RAG-1* is a major *V-(D)-J* recombinase component). In support of the second possibility, recent evidence suggests that the SCID gene may encode a protein more generally involved in DNA repair¹⁰.

Although a transfected *RAG-1* gene activates *V-(D)-J* recombination in a cell line that does not normally undergo rearrangement, it does so very inefficiently. This finding could be explained in several

ways. The most mundane is that the current *RAG-1* clone carries a debilitating lesion. A more interesting explanation is that there is a lymphoid-specific component of *V-(D)-J* recombinase in addition to *RAG-1*, and that component must also be expressed for complete recombination activity to occur. If the second possibility were true, it would point to the presence of another gene encoding an activity that would complement *RAG-1* to yield higher efficiencies in the *V-(D)-J* recombination assay — the *RBP-2* gene will be an obvious candidate to test in this context.

There are many other important questions that should readily be answered with the reagents at hand. It is essential to know whether *RBP-2* actually has recombinase activity and whether *RBP-2* gene expression and/or *RBP-2* binding activity is restricted to cells with known *V-(D)-J* recombinase activity. With respect to the various possibilities outlined here, does *RAG-1* activate expression of *RBP-2* (which may then act alone or together with other factors to mediate *V-(D)-J* recombination), or does *RBP-2* act synergistically with *RAG-1* to carry out recombination? If either or both *RAG-1* and *RBP-2* are components of *V-(D)-J* recombinase, then an *in vitro* system to study *V-(D)-J* recombination may finally be feasible. Such a system should allow complete elucidation of the mechanism of the *V-(D)-J* recombination process (for example, the nature of the 12/23 joining restriction). But it remains to be seen whether the availability of *V-(D)-J* recombinase genes and gene products will provide a system better to address the nature of the mechanisms that direct a common *V-(D)-J* recombinase to join different Ig and TCR loci (with similar or identical RS sequence) in a stage- and lineage-specific manner³. Finally, it will be important to determine how the *RAG-1* and *RBP-2* genes evolved and whether these sequences will allow identification of related, but as yet undescribed, proteins that could act in other recombinational processes. □

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Lifeless prose

JUST as the calculator has produced its own brand of innumeracy, so the word processor is generating its own style of illiteracy. Its speed and ease, says Daedalus, are filling books, papers, journals and commercial literature with increasingly muddled English.

Spelling checkers, and even the more recent grammar-sensitive 'style checkers', only give the muddle a little extra polish. The reader is still left with the thankless task of trying to make sense of it all. So DREADCO programmers are developing a novel word program. It tries to deduce what, if anything, the author has to say, and then nags him into saying it clearly.

DREADCO's 'Literary Critic' seeks clarification of every sentence typed. "You haven't mentioned protons before", it might remark; or "You said the opposite in line 157", "Is 'aromatic' the smell or the chemical?", and for real puzzles, "Do you really mean that?"

These seemingly intelligent interjections stem from the program's attempt to find a 'logical tree' behind the growing narrative. Like other conversational programs, the Literary Critic has a big dictionary of words and synonyms classified by type — animals, locations and so on — and connected by their most likely relationships. Once it has decided what the text is 'about', it queries every apparently new or discordant topic, and amends its dictionary accordingly. It marks occurrences with their stated sequence in time, keeps tabs on the 'cast' of concepts or characters being discussed, and asks further questions when ambiguities or inconsistencies seem to occur. Where logically necessary, it generates new linking text, and tries to arrange the whole assembly of statements into their neatest logical tree. Such is the power of persistent Socratic questioning, says Daedalus, that the most complex and rambling chain of remarks should soon be clarified into a coherent story. It won't be immortal prose, but it should be a grammatical and effective piece of writing.

Self-conscious stylists will greatly resent the Literary Critic with its endless silly questions. But the children and students of the electronic age will simply accept it as an effortless and automatic way of working out what they want to say, and then saying it in a straightforward manner. And many humbler folk, with fascinating ideas and personal stories to tell if they only dared to sit down and write them out, will be grateful for its tireless and patient tuition. It will help policemen and insurance assessors to extract sensible evidence from befuddled witnesses, tease effective instructions out of skilled but inarticulate craftsmen, and trick unwary criminals into much completer confessions than they had intended.

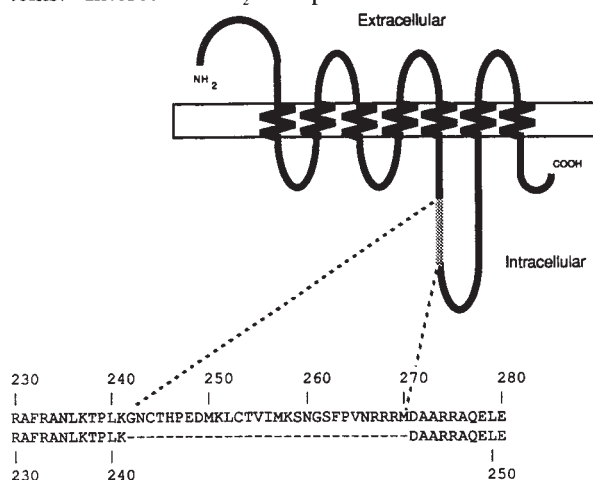
David Jones

D₂ receptor, a missing exon

SIR—A DNA sequence encoding a dopamine D₂ receptor in rat brain has recently been determined by Bunzow *et al.*¹. This sequence encodes a protein of 415 amino acids which is found in brain and anterior pituitary and is a member of a family of receptors which are coupled to G proteins. Interest in D₂ receptors stems

receptor is encoded by multiple exons¹.

Using oligonucleotide primers corresponding to sequences upstream and downstream from this portion, we found by PCR that the larger form of the dopamine D₂ receptor predominates in both rat pituitary and brain cDNA. In neither tissue did we observe the smaller form.



Schematic diagram of the dopamine D₂ receptor showing the location of an additional exon in the third cytoplasmic loop. The amino-acid sequence of the exon is shown in single letter code above that of the previously published structure¹.

largely from their involvement in the pathology of neurological and psychiatric disorders such as parkinsonism², schizophrenia^{3,4} and drug addiction⁴.

Using a cloning strategy based on the polymerase chain reaction (PCR) and oligonucleotide primers corresponding to consensus sequences of the third and sixth transmembrane segments of this gene family⁵, we have obtained several clones from a rat pituitary complementary DNA library encoding the dopamine D₂ receptor. Sequence comparisons showed that these clones were consistent with the published structure except for an additional 29 amino acids in the third cytoplasmic loop inserted after Lysine 241 (see figure). Additional analysis of genomic DNA indicated that this insert is encoded by a separate exon between the flanking sequences reported by Bunzow *et al.*¹. This region of the receptor is involved in G-protein coupling and varies dramatically between different receptor subtypes⁶. There is also considerable evidence that the dopamine D₂ receptor can couple to either the cyclic AMP or the phosphoinositide second messenger pathways, or to both⁷. It is possible that the additional exon confers an alternative G-protein specificity on the D₂ subtype, through differential splicing of messenger RNA. Although receptors of this type are normally encoded by a single exon, the D₂

The northern analysis of RNA transcripts in brain and pituitary illustrated by Bunzow *et al.*¹ could therefore represent the larger or both forms of the receptor.

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Exxon Valdez bird toll

SIR—On 24 March 1989, the oil tanker *Exxon Valdez* spilled 260,000 barrels of Alaska North Slope crude oil into Prince William Sound. Oil drifted in a south-westerly direction into the Gulf of Alaska and eventually covered 25,000 km² of coastal and offshore waters occupied by more than half a million marine birds. As predicted^{1,2}, we have witnessed an unprecedented toll of marine birds from oil pollution, which is summarized here.

Dead birds found on beaches and floating in open waters were retrieved by fishermen under contract to Exxon Oil Company, volunteers, and personnel from the US Fish and Wildlife Service (USFWS), Alaska Department of Fish and Game, International Bird Rescue

Center and other organizations. Oiled birds were processed and identified (when possible) by USFWS biologists. Data presented here (see Table) include birds retrieved between 25 March and 25 September, 1989.

Preliminary analysis of wildlife surveys conducted before^{2,3} and after (USFWS, unpublished data) the spill indicates that about 600,000 marine birds were present in areas contacted by oil. About half that number comprised species with high vulnerability to oil (such as guillemots). More than 35,000 dead birds (89 species) were retrieved from affected areas by 25 September 1989 (see Table). However, the 5,000 deaths — mainly of kittiwaks, puffins and shearwaters — in August and September, most are the result of natural causes. Most birds (90 per cent) were killed outside Prince William Sound in the Gulf of Alaska, and the relative composition of oiled birds varied markedly between those areas. In Prince William Sound, proportionally more coastal species were killed. In the Gulf of Alaska, common guillemots were most affected, with few other species comprising more than 2 per cent of the total kill. Species killed in large numbers relative to their local densities included yellow-billed loons, harlequin ducks, pigeon guillemots, marbled murrelets and bald eagles.

Based on the results of corpse-drift experiments conducted elsewhere^{4,5} and numbers of birds at risk, we tentatively conclude that the number of birds retrieved represents 10–30 per cent of the actual kill, which was probably between 100,000 and 300,000 birds. The lower estimate is conservative because outside Prince William Sound, logistics, weather and geography prevented a complete and timely search of affected areas. On the other hand, the upper estimate is probably high as it implies an almost total loss of populations at high risk — which we did not observe.

Few, if any, oil spills have had as large an impact on marine bird populations as the *Exxon Valdez* spill. Well-documented^{6–8} large oil spills, such as those from the *Torrey Canyon* (7,815 birds retrieved out of an estimated kill of 30,000), *Hamilton Trader* (4,092 out of 10,000), and *Amoco Cadiz* (4,572 out of 20,000), have rarely resulted in retrievals or estimated losses of more than 20,000 birds^{9,10}. A few lesser-known incidents of acute oil pollution in the North and Baltic seas have resulted in estimated losses of 30,000–50,000 birds, usually alcids and seabirds^{11–13}. On a global scale, the northern Gulf of Alaska harbours enormous populations of marine birds and so the magnitude of losses from the *Exxon Valdez* oil spill was predictable^{1,2}, and, not surprisingly¹, exceeds any other record of oil-related mortality we can find. If the spill had occurred in summer or autumn, the toll could have

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Total numbers and species composition of birds retrieved from affected areas after the Exxon Valdez oil spill

	Area		Total
	Prince William Sound	Gulf of Alaska	
No. retrieved	3,360	31,919	35,279
No. identified	2,884	29,468	32,352
Percentages:			
Loons and grebes	20.5	0.9	2.6
Procellariids*	0.5	14.4	13.2
Cormorants	16.0	1.3	2.6
Seaducks	24.8	2.3	4.3
Gulls	1.7	6.3	5.9
Guillemots	15.1	64.9	61.7
Other alcids†	17.2	9.7	9.1
Other birds	4.2	0.2	0.4

* Includes fulmars, shearwaters and storm-petrels.

† Includes pigeon guillemots, murrelets, auklets and puffins.

been much higher⁴. A few other locations in North America have the potential for similar bird losses, and many are the object of oil and gas exploration or development (for example, eastern Canadian Arctic, Grand Banks of Newfoundland).

Whether bird losses from the Exxon Valdez spill represent biologically significant losses in Alaska or will even be detectable in most populations remains to be seen. It will take years and even decades for some populations to return to pre-spill numbers^{14,15}, but other natural and artificial perturbations may obscure the effects of, or recovery from, oil mortality^{7,9,16,17}.

Bacterial zipper

SIR—An in-phase repetition of a leucine every seven residues in an α -helical structure is a motif associated with the dimerization of proteins and, together with an adjacent basic region, is responsible for the interaction of eukaryotic regulators and specific DNA sequences. This structural and functional motif has been termed the 'leucine zipper'^{1,2}. It is also present in membrane proteins that do not bind to DNA³⁻⁵.

In the course of our work on a new plasmid of *Pseudomonas savastanoi* (Nieto *et al.* in preparation) we have found that its replication protein (RepA) has a putative leucine-zipper motif in a sequence located at the N terminus. The computer

Furthermore, even though we suspect that certain colonies were hard hit by oil, we were unable to identify where dead birds originated, and losses may therefore be spread over a larger geographical range than we surmise⁶. In any case, local populations may recover in 20–70 years, and the process will be accelerated if birds emigrate from unaffected colonies¹⁴⁻¹⁷.

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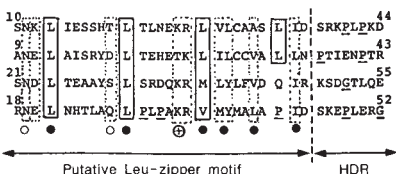
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predictions of secondary structure give this region a potential α -helical character. There are no residues incompatible with α -helix among the first four Leu residues but the two prolines that flank the fifth Leu residue should destabilize the α -helical structure; we therefore predict that the α -helix has about seven turns.

Comparison of this region of the RepA protein with known sequences of functionally equivalent proteins involved in initiation of replication in plasmids of Gram-negative bacteria, indicates that some of them⁶⁻⁸ also have a potential leucine zipper motif at the N₂-terminal region. The eventual absence of a leucine or a compatible residue at the fourth position and the presence of destabilizing residues could indicate, however, that all

Comparison of N-terminal regions of four plasmid replication initiator proteins. Coordinates of initial and terminal residues are indicated. □, Leu repeats and other compatible residues; ○, other conserved positions. Average chemical character of residues: ●, non-polar; ○, polar; ⊕, positively charged. Underlined: helix-destabilizing residues. HDR: helix-destabilizing region.

Protein	Plasmid	Ref
RepA	pPS10	44
RepA	pSC101	6
E	F	7
π	R6K	8



Which Haldane?

SIR—Mark Williamson and Robert May (*Nature* **341**, 695; 1989) in considering the Haldane beetle story appear to err by attributing it to J. B. S. Haldane, the famous geneticist. Its origin is more likely to be his father, J. S. Haldane, the distinguished physiologist, who began his work in the physiology laboratory at Oxford in 1887, or possibly his uncle, R. B. Haldane, who was Minister of War before becoming Lord Chancellor in 1912. In 1915 R.B. Haldane was excluded by Asquith from his coalition government because public opinion considered him pro-German because of his well-known enthusiasm for Kant and other German philosophers. This enthusiasm he had imparted to his young brother. Either J. S. or R. B. Haldane, as young men, might well have approached Jowett for advice on philosophy and been invited to dinner at high table at Balliol where the conversation, now a legend, could have taken place.

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these sequences may represent a leucine zipper-like motif in prokaryotes in which shorter α -helices could also be functional.

Note that the reported initiator proteins share the properties of binding to DNA and of being transcriptional regulators of their own synthesis; it has been proposed that they also interact within themselves and with other proteins of the replication machinery of the cell^{9,10}.

A computer search for the DNA-binding helix-turn-helix motif¹¹ in RepA found a C-terminal region that could fit that structure. Similar observations have been reported for the other initiation proteins^{12,13}. The N-terminal leucine zipper-like motif described here may, therefore, be involved in protein-protein interactions.

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Half-hearted exorcise

John Morton

No Ghost in the Machine: Modern Science and the Brain, the Mind and the Soul. By Rodney Cotterill. Heinemann: 1989. Pp. 341. £17.50.

RODNEY Cotterill has written a sort of anti-Thatcherian tract. He claims that dualism is the currently standard view of the mind-body relationship, and finds (p. 4) that the associated "assumed freedom of will is altogether too convenient a device for letting us justify the belief that equality of opportunity is all that should be required of an equitable society". He comes down firmly on the side of determinism, with a mechanistic view of the mind. Nevertheless, he deems it prudent "to acknowledge that such points of dispute as the existence and nature of God, and perhaps also the creation of the universe, lie fundamentally outside our understanding" (p. 8). Nonetheless, Cotterill wishes to "explode the myth of the soul". In relation to this, I should, perhaps, declare myself as a more prudent mechanist.

Cotterill's targets are religion and the law. We learn that 150 years ago one could read in a medical textbook that mental illnesses were "the sinner's retribution at the hands of God, and were caused by deprivation of free will" (p. 217). Further, "It might seem that the legal establishment would grind to a halt if it could be proved that the idea of free will is fallacious." (p. 280). In pursuit of such targets, Cotterill discusses the genetic constitution of risk-takers who have "a regrettably high probability of ending up in gaol . . . Incarcerating one of these unfortunates in a penitentiary, and condemning him to a life of inactivity, is not only grossly unjust; it is biologically unsound." (p. 177). He also discusses patients suffering from general paralysis of the insane, characterized as capable of nothing but stereotyped reactions and "docile to the point of immobility". They have clearly lost their free will, but was the loss sudden or gradual? A sudden loss would have to be reconciled with the continuous loss of neural function. A gradual loss of free will, on the other hand, would imply that "wills are Orwellian: all are free but some are more free than others" (p. 280).

So far, so good. But these should have been the starting points of Cotterill's arguments rather than robust interludes. Most of his book turns out to be a pop introduction to current work in human biology: the latest in genetics, neurophysiology and the like. For example, Cotterill discusses studies that "have revealed a strikingly unromantic picture of the nervous system" (p. 79). There is no mention of who does have a romantic view of the

dendrite; through most of the book the reader has no notion as to the nature or origin of the arguments supposedly being countered.

As strange is Cotterill's omission of cognitive psychology. Indeed, I suspect he is an eliminative reductionist; that is he would prefer to dispense with the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Winston Smith (Edmond O'Brien) struggles for free will in 1984.

psychological level as well as the spiritual. He does manage to report some of my own unpublished work (and gets it correct!), but otherwise his coverage of the field is unimpressive. On the topic of autism, for example, he refers to two books published in 1971 and one in 1972. Things have moved along considerably since then and it isn't true that "the most striking feature of the autistic patient is his or her lack of desire to originate any activity, however modest" (p. 171). Or that people with autism "seem to live within an invisible shell, incapable of interacting with their surroundings" (p. 227). Although the best currently available book was too late for Cotterill (*Autism*, by Uta Frith; Blackwell, Oxford 1989; see review by Peter Bryant in *Nature* 341, 395; 1989), there has been ample recent work stressing the communicative nature of the autistic problem. In fact, Frith's position anchors autism more firmly to a biological basis than does Cotterill's outdated characterization. The proper explanatory options of the autistic problem are not to do with will (although there is no idea too silly to find proponents), but are to do with the alternative social-emotional or psychological learning theories.

Many readers will be surprised to learn

that "Anatomical and physiological investigations, undertaken during recent decades, have elucidated the roles of almost all of the brain's components." (p. 54). On the neural underpinnings of language, Cotterill relies on a view that could have come from the nineteenth century, and he would not pass a first-year exam with the phrase "the innate language structures . . . are possessed by all except those who are unfortunate enough to have a speech impediment" (p. 197). Given such rubbish, I begin to doubt Cotterill's accuracy in the areas I am not familiar with.

Towards the end of the book a possible target for Cotterill's deeper concerns emerges in his reports of the experiments by Benjamin Libet and his colleagues who showed, essentially, that judgements of the instant of stimulation of a subject's skin were referred back to 450 milliseconds before the conscious response could have occurred. Libet found these results suggestive of dualism, and Eccles and Popper, in their book *The Self and its Brain* (Springer, Berlin, 1977) claimed "This antedating procedure does not seem to be

explicable by any neurophysiological process." This is a serious challenge to Cotterill's position, one which he explains by the notion that "decisions could be programmed subconsciously". In this he claims affinity with Freud (but he should, of course, have referred to the unconscious). "The idea that there is more going on in the brain, at any instant, than is directly orchestrated by consciousness might seem difficult to swallow." (p. 268). This is the moment of Cotterill's revelation! Some cognitive psychological discussions of the nature of consciousness would have been a short-cut to this conclusion. But Cotterill includes little or nothing of such work, nor of the appropriate philosophy, nor modern theology, all of which should have a place in a tract with these pretensions.

Cotterill's last sentence is a fitting climax to his loosely argued work: "If there is a hereafter, what am I actually going to do with myself for the next umpteen billion years?" Does he not fear he will have to repent? □

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Making a show of it

Carole Stott

For Instruction and Recreation: A Centenary History of the Museums Association.

By Geoffrey Lewis. Quiller Press, 46 Lillie Road, London SW6 1TN: 1989. Pp.98. £9.95, \$16.

MUSEUMS in Britain have reached a watershed. Gone are the days when every city proudly supported its own museum, and industrial benefactors insisted that entrance should be forever free; gone too are the days of tightly packed showcases when children were told to "be quiet".

promotional activities are only the public face of the museum world. Has anything really changed behind the façade? The Museums Association originated in the 1880s when a handful of curators met to try to develop the full potential of museums. The spur was the recent Education Acts and the shackle the miserly one-penny rate that cities were allowed to spend. The original members of the association were concerned both about inadequate financial support and the aims of their institutions. Education was high on their list of priorities, and changes in display techniques and a circulating school-loan system were their first initiatives. Training and 'proper salaries' for curators were proposed, as well as schemes for cataloguing and sharing knowledge of museum collections.

But museums have become victims of their past successes. Today's visitors are more sophisticated and less easily impressed than were their grandparents. Expectations of a museum visit have changed.

To mark the centenary of the Museums Association, Geoffrey Lewis has produced a slim volume that charts the progress from those early days to the present. The style and format are redolent of the modern easy-to-read 'museum speak' label. Each new subject is appropriately headed and self-supporting — early membership; annual conference; the diploma; legislation; the association today — and there is little here to provoke thought or debate. Museum libraries and diligent curators should have copies of the book (the bibliography alone is worth the asking price), but it is hard to imagine that it will catch the attention of a wider audience. And that is a shame.

The Museums Association has surely missed a golden opportunity to help in making itself more accessible to the general public. Centenaries are times both to reflect on the past and point the way to the future. Why isn't the Association blowing the fanfare of success, and celebrating everything that is good in the museum world? Perhaps its members realize only too well that the concerns of the 1880s are still here in the 1990s. The financial cake is small and the largest slice

seems more often to be spent on stopping the rain coming through the roof of the building — often a museum-piece in itself — than on enhancing and studying the collections that it houses.

What do we want from our museums, and from their curators? How do museums fit into the fabric of contemporary life and educational policies? How much are we prepared to spend? These questions need thoughtful answers, sooner rather than later. □

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Inside story

Richard W.E. Watts

The Metabolic Basis of Inherited Disease, 6th edn. Edited by Charles R. Scriver, Arthur L. Beaudet, William S. Sly and David Valle. McGraw-Hill: 1989. Two volumes, pp. 3,006 plus index. \$215, £155.

THE first edition of this book, published in 1960, broke new ground and soon established itself as a major work of reference for clinicians, biochemists, pathologists and an increasing range of biomedical investigators. It is now a classic, recognized, respected and, above all, widely read wherever problems in relation to the "inborn errors of metabolism" or, to use the parlance of the late twentieth century, "molecular medicine", arise in the clinic or the laboratory.

Judged against this background, Scriver, Beaudet, Sly and Valle had a difficult furrow to plough. They have done it well, digging it deep and straight to produce an up-to-date, comprehensive book which, however, is still clearly recognizable as the sixth-generation descendant of Stanbury, Wyngarden and Fredrickson (latterly plus Goldstein and Brown). In this new edition, there are many new contributors, 31 new chapters and major revisions everywhere. Those who are fortunate enough to own the earlier editions should not discard them, as the editors wisely asked their contributors to regard the earlier editions as archives for the older material. Furthermore, the comparison of individual chapters in the different editions provides a fascinating view of how, where and when knowledge in the field has moved forwards.

As in the previous editions, the material in the new volumes is largely organized around particular diseases, individual chapters being preceded by useful summaries. The discussions of the areas of biochemistry relating to a specific disease, or groups of diseases, are detailed, authoritative and well-focused. Genetic and

IMAGE
UNAVAILABLE
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REASONS

Victoria and Albert Museum, London — inappropriate exposure.

On average, a new museum opens in Britain every two weeks. Competition is now the name of the game. Museums are forced to welcome everyone; they are in the show-and-tell business. All the senses must be catered for in a 'hands-on' experience, not least taste — a priority is getting the punter into the café and gift shop with its chocolate bars, jars of jam and key-rings all incongruously placed alongside miners' lamps or dinosaur kits.

The 1990s-style exhibit displays and the

molecular-genetic information is usually introduced at this point, followed by the clinical aspects, including at least the principles of treatment. The general sections have been considerably expanded to take account of the needs of students at all levels, as well as of scientists and clinicians working in the field, to be conversant with many areas of molecular biology, cell biology, immunology, major chromosomal disorders and cytogenetics.

The field covered by the book has expanded greatly as more single-enzyme defects have been defined and as the genetic heterogeneity of those already known has been documented. Heterogeneity within the inborn errors of metabolism also stems from the fact that the specific mutation operates against different backgrounds of polygenic influences and environmental factors in different individuals. There are also genetic factors contributing to diseases that are not inherited in a simple mendelian manner but that operate through biochemical mechanisms. These considerations indicate further expansion in the future and emphasize the masterly degree of compression which has been achieved in this new edition.

Appropriately, most writers on inherited single-enzyme defects and human biochemical variation, including the present editors, look back to Sir Archibald Garrod's Croonian lecture "Inborn Errors of Metabolism", delivered before the Royal College of Physicians of London in June 1908, as a landmark in the development of their subject. The present growing confluence of the biomedical sciences, providing a substratum for our wider understanding of disease, but defying the old categorizations such as biochemistry, molecular biology, genetics and pathology, was perhaps foreshadowed in Garrod's essay "The Inborn Factors in Disease", published in 1931. In this undeservedly little-known work, Garrod explored the concept of diathesis (a condition of the body which renders it liable to certain diseases) and tried to discern some elements of the scientific basis for it.

As the last decade of the twentieth century dawns, it seems reasonable to suggest that macromolecular structure interactions and aberrations (macromolecular pathology) are the scientific bases for diatheses, and a type of explanation towards which Garrod was groping more than 60 years ago. The sixth edition of *The Metabolic Basis of Inherited Disease* points forward in this direction and hence towards a wider brief for future editions. □

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The practice of theory

M. B. Usher

Mathematical Biology. By J. D. Murray. Springer-Verlag: 1989. Pp. 767. DM89, £35, \$59.

In a minority of books, the author strikes the 'right' note and the reader says something like "I couldn't put it down". Most books do not fall into this category even when the author has something important to say. Other books, usually works of reference, simply defy reading because of the sheer magnitude of the task. Murray's book falls into this category. That is not to belittle the book in any way, as it is clearly a *magnum opus* on the application of mathematics in biology. Nevertheless, I have dipped into it rather than reading it from cover to cover, omitting some sections and rereading others. In the areas of biology that interest me, I have discovered here some fascinating analyses.

The book is divided into 20 chapters, starting with ecology (growth of single-species populations) and ending with epidemiology (here meaning the geographical spread of diseases, although the definition could also include the spread of invasive species). The beginning and end do, however, obscure the pathway that Murray takes from one to the other. Perhaps it could be said that the continuous growth of single-species populations produces the simplest model ($dN/dt \propto N$) and hence is the best starting point. Murray then expands this simple model into discrete population models for single species and into both continuous and discrete models for interacting populations. To me, the surprising feature of this section of the book is that it is set in a calculus mould; Murray does not include the equivalent formulation of discrete models as Leslie matrices and the subsequent developments of difference equations.

Murray then moves from populations to reaction kinetics, which are introduced with a detailed analysis of Michaelis-Menten theory. Biological oscillators, feedback control mechanisms and the Hodgkin-Huxley theory of nerve membranes serve as an introduction to a section on the many facets of oscillators. As might be expected, this section includes a discussion of limit cycles and of local/global stability, but it also includes topics such as unexpected 'black holes' in this area of biology.

Oscillator-generated wave phenomena are used to introduce the study of biological waves; together with the concept of reaction-diffusion the book then explodes into a bewildering array of applications,

cutting right across the traditional divisions in biology. Models of waves are used to explore the spread and control of insect populations, the calcium-activated waves across the eggs of amphibia and a teleost fish, and patterning in cultures of slime mould. I found the analyses in chapter 15 particularly fascinating because the reaction-diffusion mechanisms are used to explore mammalian coat patterns (hence the spotted leopard on the cover of the book) and, more excitingly I felt, the patterns on butterfly wings. Although some, perhaps most, patterns of lepidopteran wings can be modelled on the basis of a diffusing morphogen, it is salutary that a series of butterfly wing patterns is illustrated for which models have yet to be developed. Is this a project for a PhD student?

Pattern is not manifest solely in the morphological patterns of mammals, insects or marine algae, but also in relation to neurobiology and development. Although in chapter 16 Murray largely concentrates on drug-induced hallucinations, he also explains mathematically how sufferers of migraine headaches see geometrical patterns as a result of instabilities in neural activity in the visual cortex. He also applies these models to the patterns found on mollusc shells.

Murray concludes with two chapters on epidemics. The favourite old examples — black death, venereal diseases and rabies — are all there, with the inevitable addition of AIDS and other newer diseases. Murray applies the model for the spread of rabies to the so-far hypothetical example of what would happen if the disease were to reach Britain. Assuming that an epidemic starts near the port of Southampton, he predicts that the wave front will reach London in approximately two years and the south of Yorkshire, in the north of the country, in approximately four years.

Murray has produced a magnificent compilation of mathematical models and their application in biology. I am sure that I shall refer to the text frequently, though I feel uncertain about attempting the exercises that conclude each chapter. The text is a fine exposition of calculus models in the biological sciences. I do, however, have some misgivings: the first three words that I looked up in the index were 'matrix', 'fractal' and 'chaos'. 'Matrix' is not there, but to be fair neither is 'differential equation'. 'Fractals' are not there either, but 'chaos' is discussed now and again, mainly in relation to the discrete logistic model. I regret that the book is not as comprehensive as I at first hoped, but nevertheless, couched in terms of calculus, there is a wealth of fascinating examples here. □

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Making a real discovery

T. Cavalier-Smith

Prochloron: A Microbial Enigma. Edited by Ralph A. Lewin and Lanna Cheng. Chapman and Hall: 1989. Pp. 129. £42, \$53.

EIGHTY years ago, Mereschkowsky suggested that chloroplasts of green plants and red, brown and other algae evolved from differently coloured symbionts that independently took up residence inside the cells of different protozoa. Although the origin of chloroplasts from prokaryotic (bacterial) symbionts has been firmly established for about a decade, it is still very uncertain whether chloroplasts evolved several times, as Mereschkowsky argued, or only once or twice.

Mereschkowsky's theory of multiple origins was given a considerable boost in 1975, when Lewin and Cheng showed that the photosynthetic green cells symbiotic in certain colonial seasquirts living on mangrove roots, sea shells and corals were not eukaryotic green algae, as previously thought, but gigantic green bacteria. What was most exciting about these bacteria was not their exceptional size (9–30 micrometres, a size more typical of eukaryotic cells) but the fact that they differed from cyanobacteria and resembled instead green-plant chloroplasts in two important respects: replacement of the blue or red phycobilin pigments by chlorophyll *b*; and the stacking of their photosynthetic (thylakoid) membranes. After first thinking of them as blue-green algae (now more often called cyanobacteria) Lewin therefore renamed these unusual green cells *Prochloron*, and placed them in a separate division which he called Prochlorophyta.

Many people concluded that Lewin and Cheng had discovered another of Mereschkowsky's hypothetical symbionts and that green-plant chloroplasts had evolved from *Prochloron* or some other prochlorophyte, whereas the phycobilin-containing chloroplasts of red algae and Glaucophyceae had obviously evolved from cyanobacteria. Expeditions to Pacific islands to collect material to test this idea have resulted in the publication of nearly 100 papers, whose conclusions are lucidly summarized in this excellent and valuable book.

Virtually everything significant known about *Prochloron* and its symbiosis with seasquirts is included, together with beautiful colour plates of the fascinating variety of didemnid seasquirts that harbour *Prochloron*. In several cases the seasquirts clearly gain nutritionally from cultivating *Prochloron* in their bodies.

The main evolutionary conclusion to be drawn is that *Prochloron* is really not as fundamentally different from cyanobacteria as was once thought, and is probably not specifically related to the chloroplasts of green plants. Both Lewin and Stackebrandt now tend to accept the view, long argued by Chadeaud and myself, that *Prochloron* evolved from a cyanobacterium (by the loss of phycobilisomes and the associated evolution of chlorophyll *b* and thylakoid stacking) quite independently of green plant chloroplasts. Alberte suggests some possible selective advantages for these changes.

The recently discovered free-living prochlorophyte *Prochlorothrix*, to which the final chapter is devoted, also appears not to be specifically related to green chloroplasts. Because of the possibility of convergent multiple losses of phycobilisomes and origins of chlorophyll *b* (which differs from *a* in only one atom), one cannot even be sure that the prochlorophytes are monophyletic. It also remains possible, though perhaps unlikely, that green plants obtained their chloroplasts from the same cyanobacterium as did red algae but that euglenoids got theirs from a prochlorophyte. The discovery of a third unnamed type of prochlorophyte suggests that prochlorophytes could be more diverse than initially thought: even *Prochloron* should probably be split into at least three species. Atypical photosynthetic bacteria continue to be discovered sufficiently frequently to suggest that still more surprises may lie ahead.

Both Lewin and Stackebrandt touch on the problem of classifying the prochlorophytes. This group obviously belongs in the Eubacteria, not in the plant kingdom, despite Lewin's original creation of the botanical division Prochlorophyta. But the likelihood that these organisms evolved from cyanobacteria is insufficient reason to include them within the same family as cyanobacteria, as Stackebrandt suggests; that would be confusing and would undervalue their distinctiveness. The paraphyletic and exceedingly diverse class Photobacteria clearly should be abolished, as Stackebrandt implies. But instead one should treat Cyanobacteria and Prochlorobacteria (a better name than Prochlorophyta) as classes within a single new division — the Phycobacteria ('algal' bacteria) — which would include all those bacteria sharing the property of oxygenic photosynthesis with their descendants the chloroplasts. Phycobacteria differ sufficiently profoundly from purple and green anoxygenic bacteria to be a separate division.

An unusual and valuable feature of the book is the inclusion of abstracts of all the papers that have been published on *Prochloron*, which nicely complement the more discursive chapters on its cytology, biochemistry, symbiosis and phylogeny.

The editors' very practical, and sometimes amusing, hints for collecting and handling *Prochloron* complete a first-rate work, the first devoted to the Prochlorobacteria. □

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Sitting on the fence

David Lindley

Cold Fusion: The Making of a Scientific Controversy. By F. David Peat. Contemporary Books; 1989. Pp.188. \$16.95.

YOU would think that anyone writing about the cold fusion affair would be sensitive to the dangers of hasty publication. Not F. David Peat, apparently. Like Stanley Pons and Martin Fleischmann, who went public on the basis of what turned out to be highly disputable results, Peat has produced an account of cold fusion based on skimpy research and incomplete data. He also shies away from pronouncing judgement. This may be partly because his story comes to a halt during September 1989, when it was still possible (with a little effort) to imagine that there might be something to Pons and Fleischmann's claims, but is more a consequence of Peat's complete reluctance even to try to sift from the assertions and counter-assertions any measure of truth.

Peat's telling of the story adds little to what anyone who followed the comprehensive newspaper and magazine coverage will already know. Even so, Peat makes some mistakes with his chronology: he conflates the Materials Research Society meeting in San Diego, at which Robert Huggins unconvincingly defended his claim for excess heat, with the Electrochemical Society meeting in Los Angeles, at which Pons and Fleischmann similarly defended their claim for excess heat.

Nor is Peat's account of the science of cold fusion very penetrating. Two chapters of the book are occupied with routine expositions of nuclear physics, 'hot' fusion, solar energy, the chemistry of palladium and so on. Observing the quantity of theoretical ideas thrown out in response to Pons and Fleischmann's claim, Peat says "theoretical physicists and chemists appear to have no problem in accounting for cold fusion", a sunny judgement presumably based on the notion that a thousand completely wacky ideas weigh about the same as one or two moderately plausible ones. But in order to maintain

some critical distance, the author adds "only time will tell how well [the theories] stand up to a closer examination".

The problem with Peat's book is not that it is conspicuously wrong anywhere, but that it is so stubbornly uninformative. Throughout the tale there are disputes, disagreements and contradictions, all of which offer the author opportunities to dig deeper, unearth the truth and in the process bring to light some aspect of the nature of scientific discovery or the character of the participants. But at every such opportunity, Peat refrains from comment and moves hastily on. "For some scien-

tists," he writes near the beginning, "the Fleischmann and Pons announcement produced a sense of elation. But for others, it was a matter of skepticism." Seven chapters later, having followed the controversy through a number of rowdy scientific meetings and heard most of the relevant pros and cons, we reach Peat's final, balanced evaluation: "Cold fusion may lead nowhere. Or we may be standing on the threshold of something extraordinary." □

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Clock watching

Owen Gingerich

Empires of Time: Calendars, Clocks, and Cultures. By Anthony Aveni. *Basic Books*: 1989. Pp. 371. \$24.95.

"TIME, like an ever-rolling stream, bears all its sons away", runs a line in the familiar hymn. But is time really, objectively, like an ever-flowing stream, or is that just a Western cultural notion? Such is the central question of this aptly titled book from the astronomer turned anthropologist Anthony Aveni.

At the heart of Aveni's discourse are three chapters dealing with Mayan, Aztec

and Incan time. The author is obviously in command of this material, which has been close to his research interests for more than a decade. These time systems, devoid of borrowings from Western civilization, enable him to raise the "big question of human nature": given basically the same stimuli, do different people in different places all react to what they see in the same way? The answer is loud and clear: the intricately interlocking calendars of the Maya or the geometrically land-mapped calendars of the Incas are far removed from our own views of time, so wildly different that even writers of science fiction could hardly have dreamed them up.

Aveni focuses his account on these pre-Columbian civilizations by first consider-

ing a fascinating group of tribal societies — the Mursi and Nuer in Africa, the Bororo from central Brazil and the Trobrianders from New Guinea, these last with their edible timekeeping palolo worm. (These marine annelids spawn every year following the full moon in mid-autumn, allowing the Trobrianders to keep their lunar calendar in synchronism with the solar seasons.) Aveni's discussion ranges from basic biological rhythms, to the history and vicissitudes of the Western calendar, and even to a romp through modern cosmology. Although the book is somewhat uneven in quality, these topics offer an intriguing and enlightening menu for the general reader.

As a practicing archaeoastronomer, Aveni takes a decidedly sceptical view of the claims of nascent science at Stonehenge but, he asks, "Isn't there a bit of science in a ritual conducted at a time of year signaled by the passage of sunlight down an avenue or through a stone archway?". One might wish that he had evinced even more scepticism: Aveni cites reports that potato metabolism anticipates barometric pressure by two days, mentions that the Latin word *mens* signified "moon", and reckons that the Age of Aquarius will begin in AD 2137. (By my own calculations, it began well over a century ago.) It is also hard to believe his claim that the gregorian calendar reform was antisemitic.

These quibbles aside, Aveni has put together a well-written and handsomely illustrated volume. His exploration of the mysteries of time provides an eminently stimulating read, wonderfully suitable for the Christmas break. □

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New In paperback

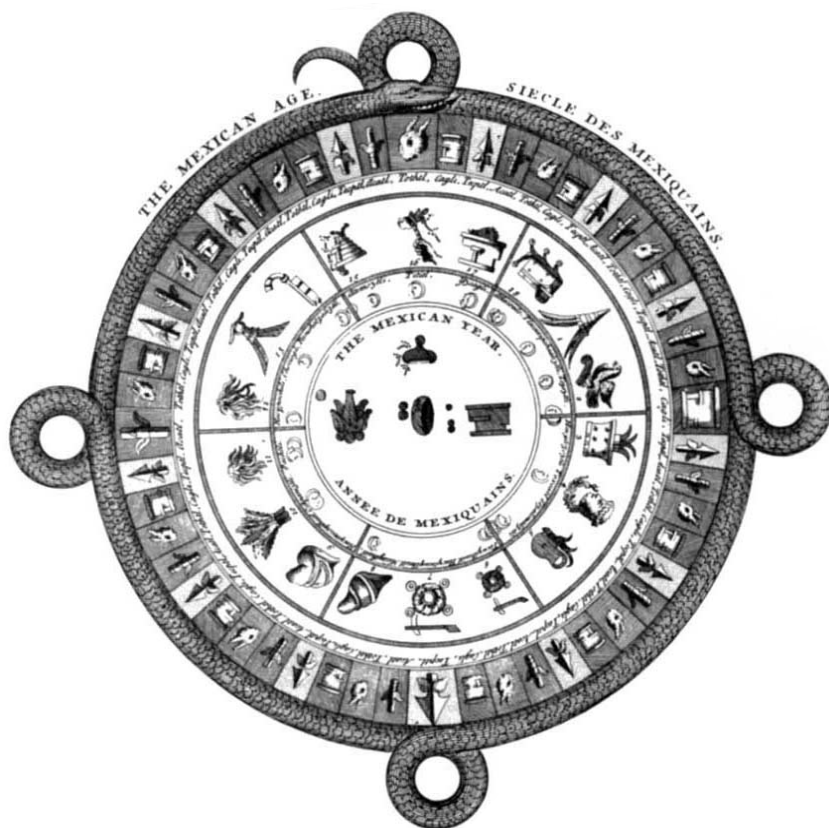
■ First published in 1988, *Time in History* by G. J. Whitrow is available in paperback. The volume discusses the general influence of time on the mental outlook and way of life in different ages and civilizations, as well as the history of the measurement of time. Published by Oxford University Press, price £5.95, \$8.95.

■ *The Dread Disease: Cancer and Modern American Culture* by J. T. Patterson is now issued in paperback by Harvard University Press, price £11.95, \$14.95 (see review in *Nature* 330, 285; 1987).

■ *Electricity and Magnetism* Volumes I and II, by B. I. Bleaney and B. Bleaney, are available as a 3rd edn in paperback from Oxford University Press. Price Vol. I £9.95, \$19.95; Vol. II £13.50, \$26.95.

■ *Universality in Chaos*, 2nd edn, by P. Cvitanović has been updated by the addition of several articles reflecting the developments in universality theory. Published by Adam Hilger, price £15.

Mary Evans Photo Library



Ancient Mexican calendar, are differences in interpretation linked to culture and civilization?

Distilled delight

D.H. Perkins

The Experimental Foundations of Particle Physics. By Robert N. Cahn and Gerson Goldhaber. Cambridge University Press: 1989. Pp. 428. £30, \$49.50.

PROGRESS in physical science depends on the close interplay between experiment and theory, and nowhere has this been more true than in the physics of elementary particles. Many standard texts on this subject tend to emphasize the beauty, conciseness and all-embracing nature of theoretical principles and ideas, so it is a pleasure to review a text in which, for a change, discussion of experiments takes pride of place.

Cahn and Goldhaber describe key experiments in twelve areas of particle physics, covering the discovery, over the years, of the various lepton and quark constituents of matter and the nature of the interactions between them. The authors have struck an excellent balance in what must have been a difficult choice of material.

The book is intended for advanced undergraduates and graduates in physics. The text in each chapter introduces a particular area of the field, presenting the basic physical concepts and background information necessary for an understanding of the significance of, typically, three or four original key papers, which are reprinted at the end of each chapter. The contents range from discovery of the neutron, positron, muon and pion in the 1930s and 1940s, to the study of "strange" particles and hadron resonances in the 1950s and 1960s, which led to the quark model and was crowned with the discovery of the massive "charm" and "beauty" states in the 1970s. There are excellent chapters on the study of weak interactions, of the strong forces between quarks, and on the discovery of neutral currents and the W and Z bosons validating the electroweak model unifying the electromagnetic and weak interactions.

I believe the authors' assessment of the role of the various experiments to be well considered, authoritative and scrupulously fair. I have to say, however, that I sometimes found the balance between the space devoted to different topics to be slightly odd. The discovery at CERN of W and Z bosons — which were a crucial prediction of the electroweak model and for which the scientific world had waited 15 years — is covered in 1½ pages of text, whereas the Stanford discoveries of charm quarks and the tau-lepton — also very important — get 20 pages. Presumably this simply reflects the north Californian origin of the authors. There are few *faux pas*, although omitting the name of Bruno

Touschek in a discussion of colliders must be one of them.

Included at the end of each chapter is a set of problems. Many of these are well beyond undergraduate level and they are mostly of a theoretical nature. What a pity that, in a text devoted to the experimental foundations of the subject, there are not more practical exercises which involve calculating a real number! My only other criticism is that a little discussion of accelerator and detector technology and development would have been useful, as these form the backbone of the experimental scene. Regarding the quality of

presentation, I have to say that an otherwise well-produced book was for me marred by the poor quality of reproduction of the original papers, particularly in the first three chapters.

In summary, I believe this text to represent a major achievement in collecting, analysing and distilling for the reader, material forming an intensely exciting chapter in modern science. □

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Spinning out

P.J. Hore

One and Two Dimensional NMR Spectroscopy. By Atta-ur-Rahman. Elsevier: 1989. Pp.578. Dfl.355, \$186.75.

NUCLEAR magnetic resonance (NMR) spectroscopy has changed almost beyond recognition in the past decade. A glance through the literature reveals a truly staggering array of ingenious and specialized techniques for determining the structures and motions of molecules. Assisted by developments in computer and magnet technology, this metamorphosis has been made possible by the ease with which long-lived coherent superpositions of nuclear spin states (coherences) can be created and manipulated using pulses of coherent, monochromatic, radiofrequency radiation.

No longer are chemists content simply to monitor the equilibrium state of the nuclear spins in a molecule subject to the restrictions of spectroscopic selection rules; rather they tailor the spectrum to suit their needs, shifting spin polarizations and coherences around the molecule so as to map out its chemical structure. A crucial factor in this revolution has been the move away from the conventional one-dimensional approach. Two-dimensional NMR, in which signals are functions of two frequency variables, is now routine, while the first forays into a third frequency dimension have occurred in the past two years. The relief of crowding in the spectra of complex molecules afforded by two-dimensional methods, together with their ability to reveal correlations between nuclei, have led to previously undreamt of achievements, most notably the determination of the complete three-dimensional structure of small proteins.

To take the greatest advantage of these new NMR techniques, the chemist needs to understand the physics underlying them. Atta-ur-Rahman's book is one of a handful of recent publications that aim to provide such insight, while also offering advice on the choice of experiment and

guidance on interpretation of spectra. The book is restricted to proton and carbon-13 NMR of liquids, and deals with essentially every significant experiment up to about 1986. An undergraduate knowledge of NMR is assumed, but not much else. Most chapters finish with exercises in spectral interpretation chosen from the author's own research in natural product chemistry.

Atta-ur-Rahman succeeds in conveying the scope and power of one- and two-dimensional NMR and shows clearly how structural information can be extracted. But he is less convincing when discussing how radiofrequency pulse sequences actually work. This he does by means of Felix Bloch's semi-classical vector model which, although simple and pictorial, cannot satisfactorily handle multiple quantum coherence or coherence transfer. As most modern techniques involve one or both of these elements, this is really a severe limitation. A newcomer to modern NMR would surely be better advised to embrace the product operator formalism to which the author devotes his final, short chapter. Once mastered, this approach requires only simple algebra and determination to enable the inner workings of complex experiments to be seen.

Sadly, there is a sufficient number of typographical errors to be irritating and, in places, confusing or misleading. Nor is the reader helped by a poorly organized introductory chapter and a rather imprecise style of writing. In short, this is a comprehensive and much-needed book, but it has serious flaws. □

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■ Two new volumes in the series *Methods in Enzymology* bear witness to the remarkable transformation in the practical application of NMR spectroscopy in the past 10 years. Volume 176, *Nuclear Magnetic Resonance* part A, covers basic techniques and methods for studying protein dynamics. Volume 177 (part B) describes protein modifications. Both volumes are published by Academic; part A is \$65, £46.50, part B (to be published in January 1990) is \$65, £46.50.

The role of mantle plumes in the development of continental drainage patterns

K. G. Cox

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The geomorphology of three continental flood-basalt provinces supports a recent model for the generation of such provinces by melting of hot mantle plumes. Characteristic drainage patterns indicating topographic doming associated with plume activity are still preserved after up to 200 Myr. Crustal thickening by magmatic underplating is the most likely cause of the persistence of such features.

THE plume hypothesis was first applied to continental areas as an explanation of flood-basalt volcanism by Burke and Dewey¹. In its new version White and McKenzie² postulate the former existence of plumes with specific sizes and locations, and it is this aspect of the model that I shall test. The model postulates that plumes of hot mantle material rise as relatively narrow columns that spread laterally at a high level to produce areas of anomalously hot asthenosphere up to 2,000 km across. When a plume rises beneath a continent the dynamic and thermal effects may produce up to 2 km of surface uplift in the centre of an extensive dome. Fracturing of the dome by gravitational forces may produce rifts and massive amounts of basaltic volcanism as the anomalously hot mantle undergoes decompressive melting. If conditions are favourable the rift may widen and a new ocean will be formed. Plume activity itself produces uplift that is strictly temporary, but even after it has ceased, the continental areas affected characteristically remain topographically high, a feature that has been ascribed to crustal thickening caused by magmatic underplating^{3,4}.

The plume hypothesis provides a framework in which many features of the geology of non-orogenic continental areas can be considered, and here I present geomorphological evidence that provides strong support for the model.

Drainage patterns over continental swells

The idea of great continental uplifts (swells) with rifted crests is an old one (see, for example, ref. 5). Figure 1 shows Cloos' drawing⁵ of the Afro-Arabian dome, split by the Red Sea, Gulf of Aden and Ethiopian rifts. This figure illustrates the fundamental components of the morphology: outwardly tilted blocks separated by rifts.

If continental break-up takes place, a typical continental flood-basalt province in a particular continent ought initially to display a morphology appropriate to (roughly) half of such a structure. The unrifted flanks of the dome should be characterized by river systems draining away from the new continental edge (dome-flank systems), whereas failed arms of the rift system should lead drainage towards the new ocean (rift-related systems). Figures 2–6 illustrate the drainage patterns of three areas of Gondwanaland affected by flood-basalt volcanism during and at the end of the Mesozoic. The circles are taken directly from White and McKenzie's diagrams and indicate their estimates of the extents of the plume tops. I believe these figures speak for themselves in showing a remarkable general correspondence between postulated plume locations and the occurrence of the predicted drainage patterns. The details (especially the extent

to which the patterns deviate from the strictly radial and the extent to which they may have been influenced by other factors such as more linear uplifts along rift shoulders or along the continental margins) will be discussed elsewhere.

The Deccan

The Deccan province⁶ (Fig. 2) is the youngest of the three, having formed at about the time of the Cretaceous/Tertiary boundary^{7,8}. The west coast of peninsular India was formed at about the same time as the eruption of the traps by the separation of India and the Seychelles bank⁹. This has been interpreted as due to a ridge jump as India passed over a plume, now believed to be situated beneath Reunion¹⁰. Deccan geomorphology and uplift have been described^{11–13}. The present western margin of peninsular India is marked by the escarpment of the Western Ghats, the northern half of which is cut into the Deccan basalts. Most rivers rise close to the west coast (sometimes within as little as 50 km) and drain eastwards into the Bay of Bengal, although in the Malwa region in the north, drainage is north-easterly into the Ganga valley. I interpret the peninsula as a whole as the flank of the remaining eastern half of the original dome. I assume that if an opposing half existed, that it has been disrupted by crustal thinning and now lies below sea level in the Seychelles and the Saya de Malha bank.

In the north, examples of exceptional drainage, towards the new ocean, are provided by the west- or south-flowing fault-controlled valleys of the Tapi, Narmada and Gulf of Cambay systems^{14,15}, which are interpreted as failed rift arms.

Southern Brazil

The coast of southern Brazil was formed in the Lower Cretaceous^{16,17} by the opening of the South Atlantic. Co-eval volcanism is represented now by the extensive basalts and rhyolites of the Paraná Basin¹⁸ (the Serra Geral Formation) and

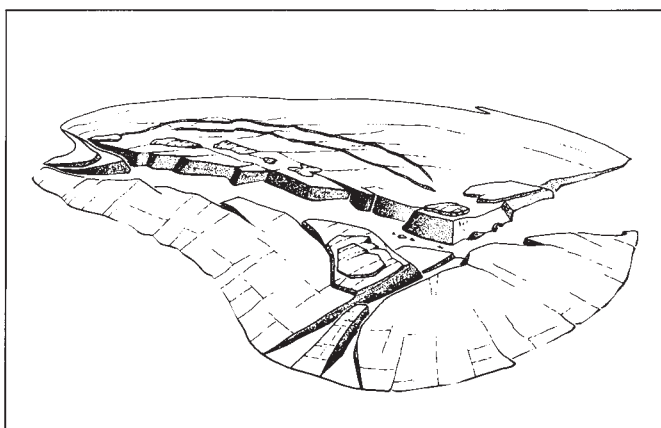


FIG. 1 The Afro-Arabian dome, redrawn from ref. 5. The view is from the south with Africa in the foreground, separated from Arabia by the Red Sea (left) and the Gulf of Aden. The Ethiopian rift system cuts the African side of the dome in the foreground. If the drawing is turned upside down a remarkable similarity is observed between the African side and the morphology of peninsular India (Fig. 2), with the Ethiopian rift playing the part of the Narmada and Tapi rifts.

by a relatively small opposing outlier on the African side at Etendeka in Namibia¹⁹ (Figs 3 and 4). In Brazil the coastal escarpment (the Serra do Mar) rises steeply for a distance of ~1,500 km between Porto Alegre in the south and Vitoria in the north, and the plateau edge inland attains heights of ~2,000 m above sea level. Geomorphologically this area is similar to the Deccan, the predominant drainage flowing westwards, directly away from the new ocean. Unlike the Indian case, however, the presence of the Andes results in the inward drainage being collected by the Paraná river system and returned to the Atlantic by the River Plate, at a point well to the south of the Paraná basalt outcrop. On the African side, only small areas of the corresponding volcanics are preserved, but there is an extensive coast-parallel topographic high, stretching from the northern part of the Cape Province of South Africa through Namibia and Angola. This directs much of the drainage away from the coast into the Kalahari Basin. I interpret the Serra do Mar and Cape-Angola topographic highs as representing the opposing halves of a single original structure.

Unlike the Deccan the Paraná dome does not seem to have been endowed with failed-rift arms.

The Karoo

In southeastern Africa the main peak of volcanism, associated with the separation of Africa and Antarctica, took place in the Lower Jurassic²⁰ and the topographic signature is more difficult to read. The area covered by remnants of the volcanism, the Karoo volcanic province, is huge, extending in the north-east from southern Malawi and the vicinity of Mozambique Island, to the north-west at Mariental in Namibia, and thence southwards over the remainder of the continent. Rocks of equivalent age and composition are found in the Dronning Maud Land area of Antarctica, where clearly the other 'half' of the province

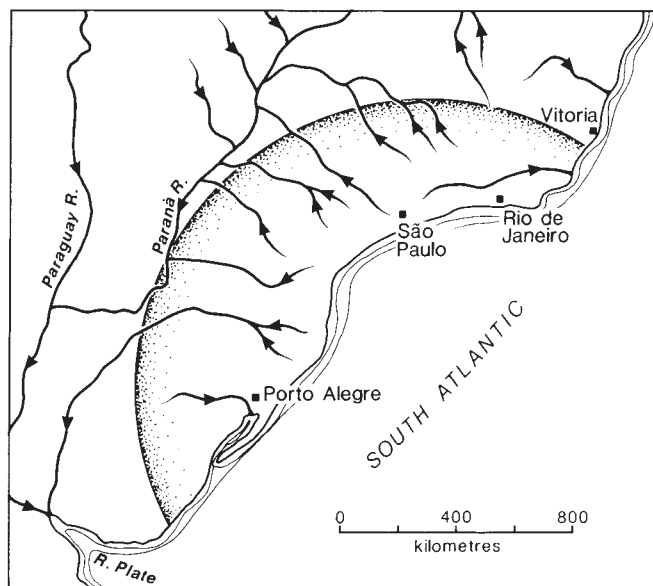


FIG. 3 Drainage pattern of southern Brazil with superimposed plume². Except in the south near Porto Alegre, dome-flank drainage is dominant.

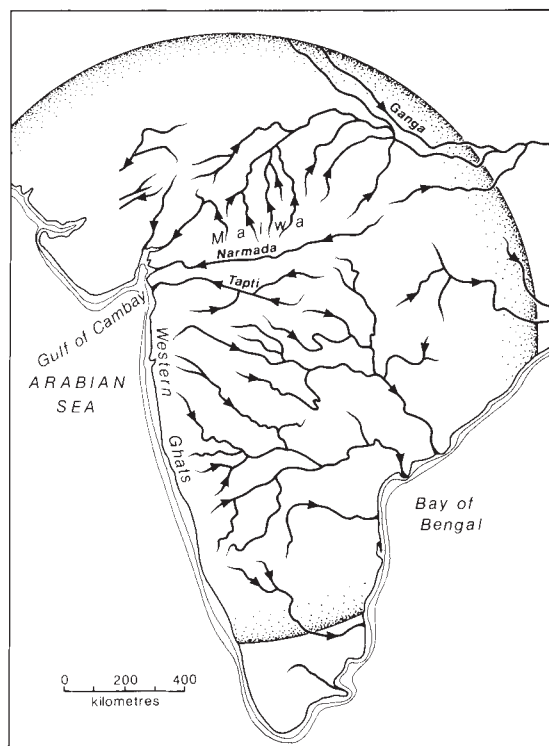


FIG. 2 Drainage pattern of peninsular India with the postulated Deccan plume² superimposed (stippled circle). Most of the peninsula preserves dome-flank drainage, also seen in the Malwa area in the north. The Gulf of Cambay, Narmada and Tapi systems consist of rift-related drainage. The westernmost left-bank tributary of the Tapi illustrates river capture of the dome-flank drainage by the rift-related system.

is located. Ice-cover, however, precludes consideration of Antarctic topography in the present work.

Because of its great extent in Africa and because of the spatial overlap of the Karoo province with the southern end of the East African rift system, it is not immediately clear which topographic features should be related to the proposed Karoo plume. High ground broadly parallels the east coast, running from Natal in the south (the Drakensberg) through the eastern Transvaal and the eastern highlands of Zimbabwe into southern Malawi and neighbouring parts of Mozambique. Maximum elevation is reached in the heavily dissected mountains of Lesotho (Thaba Ntlenyana, 3,500 m) but there are large tracts of plateau country at ~2,000 m above sea level in the Orange Free State and the Transvaal. All the high ground mentioned lies within White and McKenzie's postulated plume top.

Two major rivers, the Limpopo and the Zambezi, flow eastwards into the Indian ocean, interrupting the coast-parallel topographic high. Both are zones of Karoo-age rifting^{1,21}, and hence analogous to the Narmada in the Deccan province. The greater age of the Karoo province, however, has resulted in considerable extension of the drainage basins of these rivers, leaving the dome-flank sectors much reduced. There are still two areas, however, where the characteristic drainage patterns are well preserved.

A pattern analogous to that of southern Brazil and peninsular India is still obvious in the southern part of the province (Fig. 5). Here there are no rifts to disturb the simple pattern of westward drainage off the crest of the Drakensberg into the Orange River and Vaal River systems. The Orange River rises only 150 km from the south-east coast but flows into the Atlantic at Oranjemond, 1,400 km due west (Fig. 4), so it is evidently a classic case of drainage directed away from the new ocean. The dome-flank drainage is also excellently preserved in north-western Zimbabwe where the right-bank tributaries of the Mid-Zambezi flow with sub-parallel courses to the north-west, at right angles to the Zambezi itself (Fig. 6).

The evidence from Africa supports White and McKenzie's positioning of the Karoo plume and its areal extent. It is not obvious, however, that the Antarctic side of the plume is realistic in their presentation because it takes no account of volcanism through the Transantarctic mountains (see, for example, refs 22, 23 for further discussion).

Antiquity of drainage patterns

Major continental drainage patterns have often been postulated as being of considerable antiquity²⁴ although they are obviously difficult to date (see summary in ref. 25). Here the extremely close spatial correspondence between proposed plume locations and predicted drainage systems indicates a direct genetic relationship and, by implication, that the river systems originated at the same time as the plume activity. Thus for example, the Orange system must have originated in the Jurassic, probably at or about the time of the Lower Jurassic peak of Karoo activity²⁶ about 190 Myr BP (before present). Between the Kalahari and Oranjemond, the Orange cuts a considerable gorge through the Cape-Angola high. The river is <200 m above sea level, but is closely bounded, especially to the north, by substantial tracts of country at elevations of 1,500 m or more. The Orange is clearly antecedent to the Cape-Angola high, a conclusion consistent with the postulated Jurassic age of the drainage system, and the implied Lower Cretaceous age of the high, as the eastern half of the Paraná dome.

The Orange thus predates the South Atlantic and presumably originally flowed on into the general region of Buenos Aires, lending weight to speculation²⁵ about the possibility of other African rivers such as the proto-Congo flowing across South America.

Surface uplift

The plume model involves initial uplift of the existing land surface, in response to the dynamic effects of the plume. This may have initiated the drainage patterns observed, yet they still exist, and in all cases the postulated plumes are now far from the provinces concerned. White and McKenzie argue that further magmatic material accretes to the base of the crust during plume

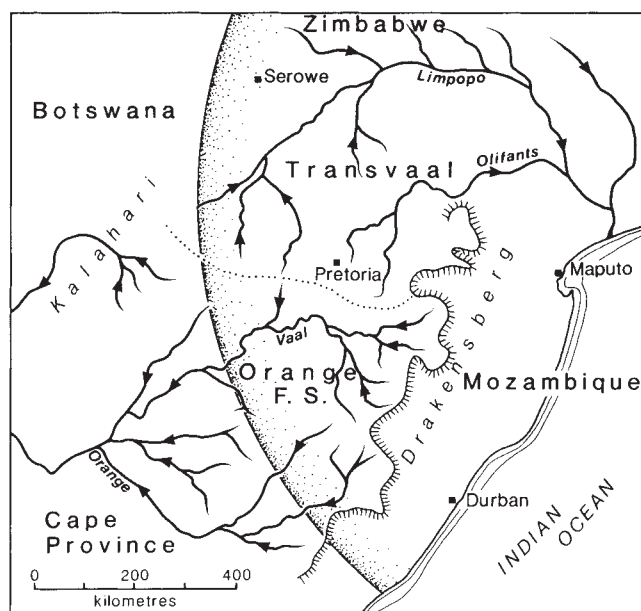
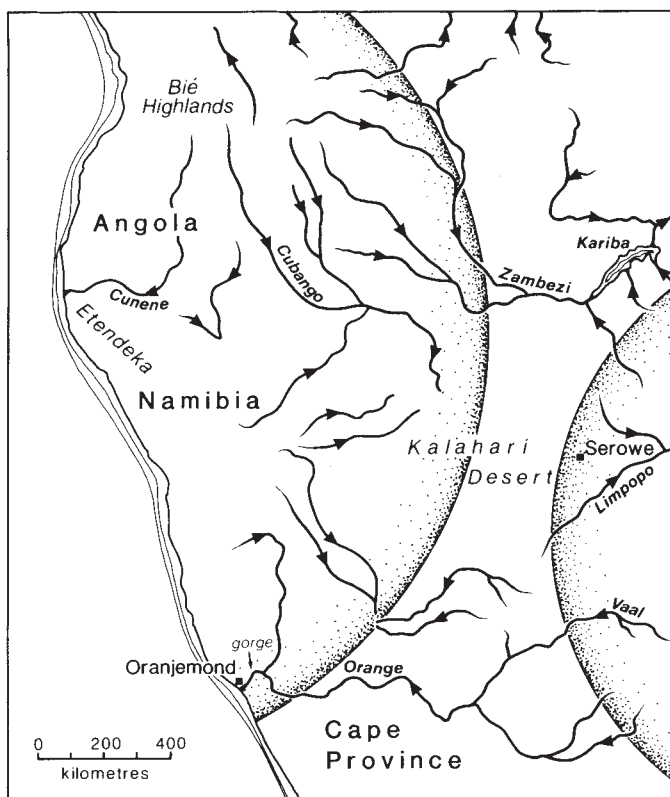


FIG. 5 Drainage patterns in southeastern Africa, with Karoo plume superimposed. Dome-flank drainage is preserved west of the Drakensberg escarpment (Orange River and Vaal systems). The dotted line separates dome-flank drainage in the south from rift-related (Limpopo) drainage in the north. Note the extreme enlargement of the catchment of the latter compared with the Narmada-Tapti system in Fig. 2.

activity (the phenomenon of underplating) and that the resulting crustal thickening causes the basalt provinces to remain topographically high in the long term. This hypothesis is based on petrological arguments²⁷ and is supported by wide-angle seismic experiments in the vicinity of rifted areas (some of which have been drilled), but it is difficult to test directly. For further studies of this problem, however, it is important to try to quantify the uplift.

Surface uplift (the term is used to mean that the average elevation of the ground increases²⁸) on a regional scale is difficult to demonstrate. Here I shall make only a very general argument, mainly for the Karoo province, that suggests the likelihood of about 1.5 km of post-Lower-Jurassic surface uplift for the Drakensberg region. The Palaeozoic history of a large part of Gondwanaland, particularly the western and southern regions (the Paraná Basin²⁹, the Karoo³⁰, and the Transantarctic Mountains³¹) was one of platform sedimentation. In the Lower Palaeozoic much of this showed marine influences, but by the Permian, deltaic and fluvial environments were widespread. By the beginning of the Mesozoic a vast area had been transformed into the subaerial desert over which the Cave Sandstone was deposited (southern Africa) and, where not terminated by volcanism, these conditions persisted right through the Jurassic into the Lower Cretaceous (for example, in the Botucatu sandstones of the Paraná Basin). The Palaeozoic/early Mesozoic history is thus one of an extensive depositional basin slowly filling up and eventually becoming a subaerial desert.

After the volcanism, the whole area has behaved completely differently, and the dispersed fragments of this region of Gondwanaland have been high-standing erosional environments. This constitutes a strong general argument in favour of long-lasting surface uplift.

I assume that the Cave Sandstone desert lay fairly low at the time of initial Karoo volcanism, perhaps <1,000 m above sea level. The sub-basalt unconformity now lies at 2,000 m in Lesotho, but more importantly, over a large area to the north of this, through the Orange Free State and the eastern and northern Transvaal, although the basalts have largely been removed by erosion, the Karoo sedimentary sequence is still

FIG. 4 Drainage patterns of the Cape-Angola topographic high, with the Paraná plume (left) and part of the Karoo plume (right). An irregular dome-flank pattern is preserved draining eastwards into the Kalahari. Note the Orange River gorge, where antecedent drainage cuts the younger uplift.

widely preserved. This is a plateau area unincised by major canyons, so the average elevation of large tracts is clear. Much of it approaches 2,000 m, and the implication of the Karoo geology is that the basalt unconformity lay not far above the present erosion surface. Returning to the initial assumption about the elevation of the desert, this indicates surface uplift of 1–2 km.

I have speculated that the Kalahari basin might owe its existence to the fact that it was unaffected by both the Karoo and Paraná volcanic episodes, hence remaining free of the associated uplift²⁰ (the 'passive basin' model¹). Much of the basin lies 500–1,000 m above sea level. There has been both post-Jurassic erosion and deposition³⁰ but I speculate that the present elevation is still about the same as that of the general pre-Jurassic land surface of southern Africa. The Kalahari is just the present-day continuation of the great Cave Sandstone and Botucatu desert, and its present elevation (about 1.5 km below the Transvaal/Orange Free State plateau) gives some support to the calculation above.

Erosion surfaces

Thus far I have tried to view the geomorphological evolution of the areas discussed in terms of consequences of the initial plume activity, which is in some cases very old. Yet the geomorphological literature of the Gondwanaland continents is rich in references to cyclical-erosion surfaces and scarp retreat^{32–34} and in many cases relatively recent, such as late Cenozoic, uplift events have been recognized. Partridge and Maud³⁴ particularly stress the episodic nature of uplift. Can the two views be reconciled? Can the whole evolution be seen in terms of a single original cause, or is it necessary to postulate a series of apparently unrelated uplift events?

A reconciliation may be found by postulating mechanical coupling between the continental crust and the newly-formed oceanic crust. After an early surface uplift has created a coast-parallel topographic high, erosion of the swell promotes rock uplift (used in the sense of England and Molnar²⁸ to indicate upward movement of bedrock) in response to denudation and isostatic adjustment. The continental margin is subjected to

opposing stresses. Inland the bedrock is travelling upwards; on the other side the oceanic crust is, if anything, likely to founder. I assume that flexure of the lithosphere can accommodate, up to a point, these conflicting demands. Beyond that point the continental margin may respond by fracture and undergo local surface uplift. Despite the fact that rock uplift is continuous in the continental interior, scarp retreat in response to episodic marginal uplift, with concomitant changes of the base level of erosion, can generate the impression of episodic uplift throughout.

Drainage-pattern preservation and plume location

After initial formation, dome-flank drainage patterns come under attack from rift-related systems. Within continental marginal zones the latter flow at low topographic levels relative to the adjacent dome-flank systems, and consequently expand their catchments by river capture. Some of the left-bank tributaries of the Tapti (Fig. 2) show this in an early stage (65 Myr after initiation), and the huge bite the Limpopo system has taken out of the Karoo dome flanks in 180 Myr is notable. Preservation is thus partly related to the age of the province, but dome-flank drainage patterns are best preserved in areas where the volcanics, or flat-lying sub-volcanic sediments, are still present or where they have only relatively recently been removed by erosion. These conditions are met by the Deccan, much of the Paraná, and the Orange Free State and Transvaal, where preservation is aided by the presence of the widespread flat-lying sedimentary rocks of the Karoo Basin. In northwestern Zimbabwe a thin veneer of basalts is still present, accounting for the very regular pattern of the mid-Zambezi right-bank tributaries.

By contrast, the Cape–Angola topographic high is deeply eroded, as the towering Jurassic/Cretaceous³⁵ plutons of Damaraland testify. Fission-track studies³⁶ indicate several kilometres of erosion during the Cretaceous. As a result the drainage system into the Kalahari basin, although still recognizable, has now adopted a more complex pattern as it adjusts to heterogeneous basement rocks. Apart from the small Etendeka remnant, whatever basalt cover was present has been completely removed.

White and McKenzie positioned their plumes using two main criteria: the centre had to coincide with the pre-drift position of a known hotspot if present, and the circle had to include the known seaward-dipping reflectors as far as possible. Here I have shown the expected drainage patterns, which support the model despite its evident simplicity. Future studies of denudation, for example by fission-track methods, and more detailed analysis of drainage patterns that are less obviously preserved, however, may enable more extensive mapping of the areas influenced by plumes. □

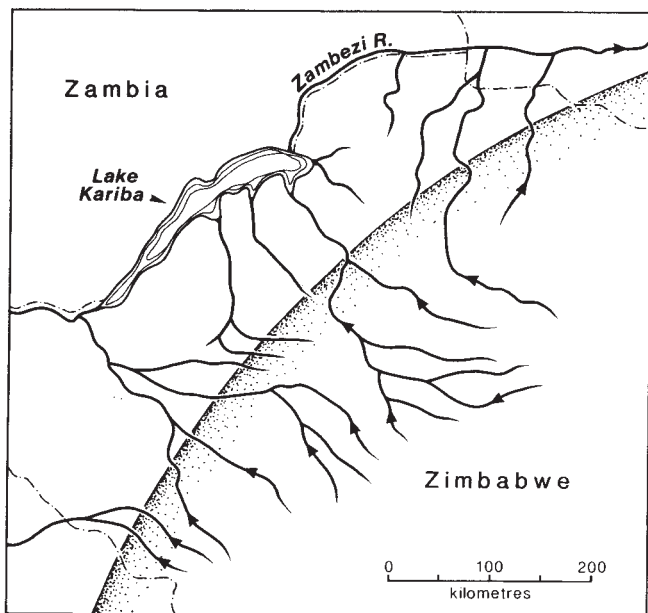


FIG. 6 Drainage in the northwestern part of the Karoo province, with part of the Karoo plume. Right-bank tributaries of the Zambezi are shown, and represent well preserved dome-flank drainage. The southeastern part of the area is occupied by left-bank tributaries of the Limpopo, the enlarged rift-related system of which the southern side is shown in Fig. 5.

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Conformations of immunoglobulin hypervariable regions

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On the basis of comparative studies of known antibody structures and sequences it has been argued that there is a small repertoire of main-chain conformations for at least five of the six hypervariable regions of antibodies, and that the particular conformation adopted is determined by a few key conserved residues. These hypotheses are now supported by reasonably successful predictions of the structures of most hypervariable regions of various antibodies, as revealed by comparison with their subsequently determined structures.

THE relationships between the amino-acid sequences of immunoglobulins and the structures of their antigen-binding sites are important for understanding the molecular mechanisms of the generation and maturation of the immune response and for designing engineered antibodies. Antigen-binding sites are formed by six loops of polypeptide, the hypervariable regions; three from the variable domain of the light chain (VL) and three from the variable domain of the heavy chain (VH), denoted L1, L2, L3, and H1, H2, H3, respectively (Fig. 1a). Within the domains, the loops are connected to a β -sheet framework whose structure is conserved^{1,2}. The specificity and affinity of the binding sites are governed by the structures of the six hypervariable regions^{3,4}.

Two models can be proposed for the relationship between the amino-acid sequence and structure of the binding-site loops. In one model, different sequences produce different conformations for both the main chain and side chains of the loops. Because hypervariable regions have different sequences in different antibodies, this model implies that each region adopts a different conformation in different antibodies. In the other

model, antibodies have only a few main-chain conformations or 'canonical structures' for each hypervariable region. Most sequence variations would only modify the surface provided by the side chains on a canonical main-chain structure. Sequence changes at a few specific sets of positions would switch the main chain to a different canonical conformation.

Canonical structure model

Experimental evidence indicates that the canonical structure model describes the relationship between amino-acid sequence and structure for at least five of the six hypervariable regions^{5–9}. Kabat *et al.*⁵ found conserved residues at sites within certain sets of hypervariable regions and suggested that they had a structural role. Padlan and Davies⁶, and more recently de la Paz *et al.*⁷, showed that some of the hypervariable regions in the immunoglobulins of known structure have the same main-chain conformation in spite of several differences in sequence.

Chothia and Lesk⁸ identified the residues that through packing, hydrogen bonding, or the ability to assume unusual values of the torsion angles ϕ , ψ or ω , are primarily responsible for the main-chain conformations of the hypervariable regions in the structures then known—the Fab fragments of NEW (ref. 10), McPC603 (ref. 11), KOL (ref. 12) and J539 (ref. 13) and the VL domains of REI (ref. 14) and RHE (ref. 15). The conformations are determined by the interactions of a few residues at specific sites in the hypervariable regions and, for certain loops, in the framework regions. Hypervariable regions that have the same conformations in different immunoglobulins have the same or very similar residues at these sites (Fig. 1 and Table 1). Examination of the amino-acid sequence of the antibody D1.3 showed that its hypervariable regions are the same size as those in known structures and contain the same or similar residues at the sites responsible for known conformations⁹. On the basis of these observations the atomic structure of the VL–VH dimer of D1.3 was predicted before its experimental determination. Comparison of this predicted structure with the preliminary crystal structure showed that the conformations of four of the hypervariable regions had been predicted correctly; the conformation of L3 was significantly different from that predicted, and

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TABLE 1 Sequences and conformations of V_K and V_H hypervariable regions of known structure

L1 Regions†																		
Canonical Structure	Protein	26	27	28	29	30	31	a	b	c	d	e	f	32	2	25	33	71
1	J539	S	S	S	*	S	—	—	—	—	—	—	—	S	*	*	*	*
	HyHEL-5	S	S	S	V	N	—	—	—	—	—	—	—	Y	I	A	L	Y
	NQ10	S	S	S	V	R	—	—	—	—	—	—	—	Y	I	A	M	Y
2	REI	S	Q	D	I	I	K	—	—	—	—	—	—	V	I	A	L	Y
	D1.3	S	G	N	I	H	N	—	—	—	—	—	—	Y	I	A	L	Y
	HyHEL-10	S	Q	S	I	G	N	—	—	—	—	—	—	N	I	A	L	F
	NC41	S	Q	D	V	S	T	—	—	—	—	—	—	A	I	A	L	Y
3	McPC603	S	E	S	L	L	N	S	G	N	E	K	N	F	I	S	L	F
4	4-4-20	S	Q	S	L	V	H	S	—	N	G	N	T	Y	V	S	L	F

Total no. of sequences known for L1 regions: human, 95; mouse, 299.

Canonical structure	1	2	3	4
Human sequences that fit (%)	—	60	5	5
Mouse sequences that fit (%)	15	25	20	10

L2 Regions

Canonical Structure	Protein	50	51	52	48	64
1	REI	E	A	S	*	*
	McPC603	G	A	S	I	G
	J539	E	I	S	I	G
	D1.3	Y	T	T	I	G
	HyHEL-5	D	T	S	I	G
	HyHEL-10	Y	A	S	I	G
	NC41	W	A	S	I	G
	NQ10	D	T	S	I	G
	4-4-20	K	V	S	I	G

Total no. of sequences known for L2 regions: human, 69; mouse, 183.

Canonical structure	1
Human sequences that fit (%)	95
Mouse sequences that fit (%)	95

L3 Regions

Canonical Structure	Protein	91	92	93	94	95	96	90
1	REI	Y	Q	S	L	*	Y	*
	McPC603	D	H	S	Y	P	L	N
	D1.3	F	W	S	T	P	R	H
	HyHEL-10	S	N	S	W	P	Y	Q
	NC41	H	Y	S	P	P	W	Q
	4-4-20	S	T	H	V	P	W	Q
	NQ10	W	S	S	N	P	L	Q
2	J539	W	T	Y	*	L	I	*
3	HyHEL-5	W	G	R	N	*	—	*

Total no. of sequences known for L3 regions: human, 52; mouse, 152.

Canonical structure	1	2	3
Human sequences that fit (%)	90	—	2
Mouse sequences that fit (%)	80	10	1

H1 had a very different fold from that predicted⁹. (We report below that the refined conformation of D1.3 corresponds more closely to the predicted structure.)

An examination of the library of the known immunoglobulin sequences shows that many immunoglobulins have hypervariable regions that are the same size as those in the known structures and contain the same or closely related residues at the sites responsible for the known conformations⁸. These observations indicate that for at least five of the hypervariable regions there is only a small repertoire of canonical main-chain conformations and that the conformation actually present can often be predicted from the sequence by the presence of specific residues.

The accuracy of the canonical structure model for immunoglobulin binding sites depends on (1) the correct deter-

mination of the sets of residues responsible for the observed conformations and (2) changes in the identity of residues at other sites not significantly affecting the conformations of the canonical structures. The model can be tested, refined and extended by using it to predict the atomic structures of binding sites in immunoglobulins before their structures have been determined by X-ray crystallography.

We have now tested the canonical structure model by using it to predict the structures of four immunoglobulins before their structures had been experimentally determined. These immunoglobulins are HyHEL-5 (ref. 16), HyHEL-10 (ref. 17), NC41 (ref. 18) and NQ10 (S.S., P.M.A. and R.J.P., manuscript in preparation). The analysis of the amino-acid sequences of these immunoglobulins indicated that 19 of their 24 hypervariable regions should have conformations close to known canoni-

H1 Regions†

Canonical Structure	Protein	26	27	28	29	30	31	32	34	94
1	McPC603	*	*		*				*	*
	KOL	G	F	I	F	S	D	F	M	R
	J539	G	F	D	F	S	S	Y	M	R
	D1.3	G	F	S	L	T	G	Y	V	R
	HyHEL-5	G	Y	T	F	S	D	Y	I	R
	NC41	G	Y	T	F	T	N	Y	M	R
	NQ10	G	F	T	F	S	S	F	M	R
	4-4-20	G	F	T	F	S	D	Y	M	G
1'	NEW	G	S	T	F	S	N	D	Y	R
	HyHEL-10	G	D	S	I	T	D	D	W	N

Total no. of sequences known for H1 regions: human, 50; mouse, 321.

Canonical structure	1
Human sequences that fit (%)	50
Mouse sequences that fit (%)	80

H2 Regions§

Canonical Structure	Protein	52a	b	c	53	54	55	71
1	NEW	—	—	—	Y	H	G	
	D1.3				G	D	G	
	HyHEL-10	—	—	—	Y	S	G	
2	HyHEL-5	*					(*)	*
	NC41	P	—	—	G	S	G	A
		T	—	—	N	T	G	L
3	KOL	D	—	—	D	G	S	*
	J539	P	—	—	D	S	G	R
	NQ10	S	—	—	G	S	S	R
4	McPC603	N	K	G	N	K	Y	*
	4-4-20	N	K	P	Y	N	Y	R

Total no. of sequences known for H2 regions: human, 54; mouse, 248.

Canonical structure	1	2	3	4
Human sequences that fit (%)	15	1	40	15
Mouse sequences that fit (%)	15	40	5	20

The residues listed here (single-letter code) are those that form the hypervariable regions and those in the framework regions that are important for the observed conformations of these regions⁹. The hypervariable regions are taken as those outside the framework β -sheet⁸. Except for H2, they are similar to, but not identical with the regions that show high sequence variations and which Kabat *et al.*²⁶ use to define hypervariable regions. The sequences are grouped so that those that have the same main-chain conformation, or canonical structure, are adjacent. The canonical structure numbers used below refer to the conformations shown in Fig. 1. The residues in the hypervariable and framework regions that are mainly responsible for these conformations⁸ are indicated by an asterisk. The classification and sequence requirements of the H2 conformations have been revised in the light of work described here and elsewhere²⁸. For each hypervariable region the number of human and mouse sequences listed by Kabat *et al.*²⁶ are given. We also give the percentage of these sequences that are the same size as the known canonical structures and have the same residues at the positions marked by an asterisk.

† Canonical structure 4 is illustrated in Fig. 4. Although the size of the known L1 structures varies between 6 and 13 residues, they have closely related folds with residues 26–19 and 32 packed against the framework in the same conformation⁸. The remaining residues form a turn or loop on the surface (Figs 1 and 4). The ends of the long loops have some flexibility. There are another 25% of the human sequences and 20% of the mouse sequences that have one more residue than structure 2, or one fewer than structure 4, and whose sequences satisfy the requirements listed above. It is expected that these differ only in the conformations of the tips of the surface loops.

‡ The H1 hypervariable regions with canonical structure 1 have very similar conformations: the r.m.s. differences in the coordinates of their main-chain atoms are 0.3–0.8 Å. The H1 regions in NEW and HyHEL-10 only partly satisfy the sequence requirements for structure 1 and have a distorted version of its conformation.

§ The H2 region here comprises residues 52a–55. The region with high sequence variation is 50–65 (ref. 26). In the known structures the main-chain conformation of 50–52 and 56–63 do not differ significantly⁸ (Fig. 1b). (*). The residues at positions 55 or 54 in the canonical structures 2, 3 and 4 have residues with positive values for ϕ and ψ , and usually, but not in all cases, Gly, Asn or Asp is found at these sites. For a sequence to match that of canonical structure 2, 3 or 4 the presence of these residues at sites 54 or 55 is required.

cal structures. We then compared the predicted structures of these hypervariable regions with the subsequently determined structures. Another immunoglobulin structure, 4-4-20 (ref. 19) has recently been reported. We did not have the opportunity to predict the structure of 4-4-20 before its experimental determination, and we discuss here only how its hypervariable regions have the conformations expected from the known canonical structures. Also, we report that the refined conformation of D1.3 (ref. 20) corresponds more closely to the predicted structure.

Model building procedure

The main-chain conformations of the hypervariable regions in the V_K and V_H domains of known structure are shown in Fig. 1. The residues responsible for these conformations are listed

in Table 1. Each hypervariable region in the immunoglobulins of unknown structure was examined to determine (1) whether it has the same size as any homologous hypervariable region of known structure and (2) whether its sequence contains the set of residues responsible for a known conformation. Except for L3 in HyHEL-5, all the light-chain regions correspond to a known canonical structure, as do all the H1 regions and the H2 region in HyHEL-10 (Table 1). The conformation predicted for the H2 regions in NC41 and HyHEL-5 was based on the analysis of the H2 region in the preliminary structure of J539 (ref. 8). In all three of these antibodies the H2 region is a four-residue turn with Gly at the fourth position and the predicted conformation is that almost always found for such turns²¹. (Below we present a more accurate analysis of H2 regions.) For H3 regions in HyHEL-5, HyHEL-10, NC41 and NQ10, no prediction of

conformation could be made on the basis of the known canonical structures.

The sequences of the VL and VH domains were compared to see which of the known framework structures have sequences close to those of the unknown structures. From the comparisons

of the hypervariable and framework regions, one known VL and one known VH structure were taken as the starting points—parents—for the model of the predicted structure. If the conformation predicted for a hypervariable region was not present in the parent, but was present in another known structure, the hypervariable region in the parent was replaced by that in the other structure. Side chains in the parent that were different from those in the unknown structure were replaced^{22,23} and the resulting model subjected to a very limited energy refinement²⁴.

HyHEL-5, HyHEL-10 and NC41 hypervariable regions

The atomic structures of Fab fragments of immunoglobulins HyHEL-5, HyHEL-10 and NC41 in complexes with their protein antigens were determined by X-ray crystallography¹⁶⁻¹⁸. The resolution of the X-ray data used to determine the structures and value of the residual (*R*) after refinement are (complex, resolution, *R* value): HyHEL-5-lysozyme, 2.5 Å, 20%; HyHEL-10-lysozyme, 3.0 Å, 24%; and NC41-neuraminidase, 2.9 Å, 19%. These structures have been determined at medium resolution. The tracing of the polypeptide chain of the hypervariable regions is unambiguous, although the orientation of some of

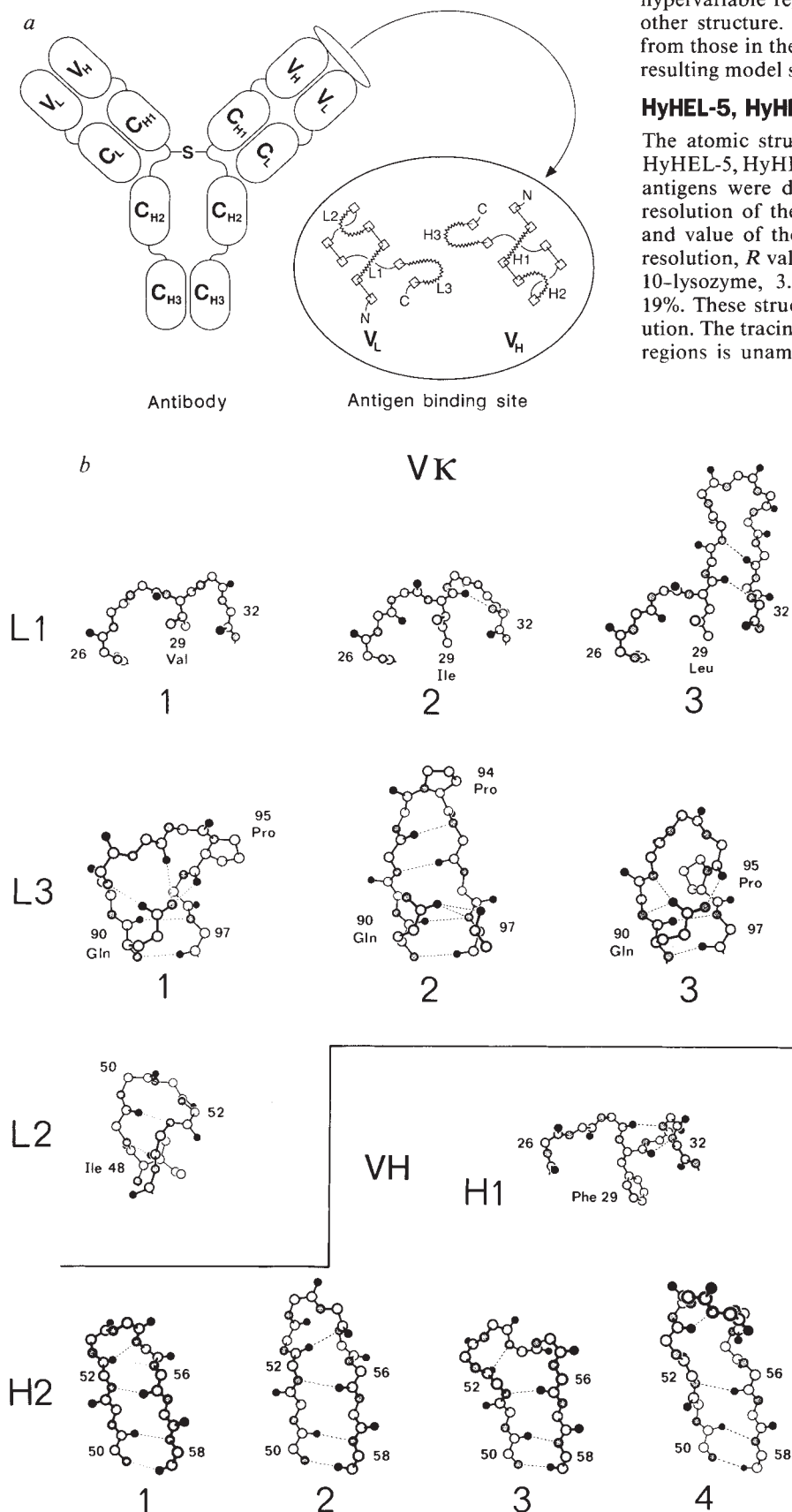


FIG. 1 *a*, Antigen-binding sites of immunoglobulins are formed by six loops of polypeptide, three from the VL domain L1, L2 and L3 and three from the VH domain H1, H2 and H3 (wavy lines). These loops are attached to strands (□) of a conserved β -sheet. *b*, Canonical structures for the hypervariable regions of V κ and VH domains. In each drawing the region is viewed so that the accessible surface is at top and the framework region below. The main-chain conformation and some of the side chains that determine this conformation are shown. For the definition of the hypervariable regions used here, see Table 1. The immunoglobulins in which the different canonical structures occur are listed in Table 1.

the peptide groups is uncertain. Most side chains are unequivocally placed.

Figure 2 shows the predicted and observed structures of each of the hypervariable regions, superposed by a least-squares fit of their main-chain atoms. Table 2a gives, for each predicted and observed hypervariable region, the r.m.s. difference in position of the main-chain atoms.

The main-chain conformations of the predicted and observed hypervariable regions are very similar (Fig. 2, Table 2a). The only exception is the H1 region of HyHEL-5. Although the observed and predicted conformations of residues 26–29 and 32 are the same, residues 30 and 31 are in quite different positions. The recently refined structure of Fab J539 (T. N. Bhat, E.A.P. and D.D., manuscript in preparation) shows that these differences were inherited as a result of an error in the original determination of the J539 structure used as the parent for this region. Rebuilding the predicted model with the refined J539 structure puts residues 30 and 31 in the correct position and gives an r.m.s. difference between the predicted and observed H1 regions of 0.6 Å.

Given the medium resolution of the structures used to derive the models and of the experimental structures, the agreement of the predicted and observed loop conformations is excellent.

Relative positions of the hypervariable regions

Figure 3 shows the positions of the hypervariable regions relative to each other and to the framework for the predicted and observed structures. To produce this figure the predicted and observed structures were superposed by a least-squares fit of framework residues. In Table 2b, the differences in position of the hypervariable regions are reported.

Small differences in the relative positions of the hypervariable regions in the predicted and observed structures might be expected because of two factors not corrected for in the model build-

ing. First, the predicted structures were built using parent V domains that have some residues in the framework and VL–VH interface that are different from those in the final predicted structure. These differences have small but significant effects on the main-chain structure of the individual domains and the way they pack together^{2,25,26}. Second, differences between the predicted and observed structures could arise from the effects of the association with the antigen¹⁸. In other proteins, ligand binding can result in the movement of close-packed segments of polypeptide relative to each other by 1–2 Å, and the ends of loops are able to move somewhat more²⁷.

The differences in the positions of the predicted and observed H2 regions in HyHEL-5 and NC41 (Table 2b) are larger than expected from these factors. The interactions that the H2 regions make with the rest of the VH domain were therefore examined.

Residue 71 and position and conformation of H2

At the same time as the structures of HyHEL-5 and NC41 became available, the refinement of the atomic structure of J539 was completed (T. N. Bhat, E.A.P. and D.D., manuscript in preparation). The conformation of H2 in the refined structure is not like that in HyHEL-5 and NC41 but is the same as that in KOL. This was quite unexpected. The main determinant of the conformation of small turns is usually the position of glycine residues²¹: in KOL, Gly occurs at position 54, and in J539, HyHEL-5 and NC41, Gly occurs at position 55.

An examination of the environments of the H2 regions²⁸ shows that in KOL and J539 the side chain of framework residue Arg 71 packs between, and forms hydrogen bonds to, H1 and H2. In HyHEL-5, residue 71 is Ala, and here the cavity that would be created by this smaller side chain is filled by the insertion of a residue from the H2 region—Pro 52a. In KOL and J539 the side chain at position 52a is on the surface. The relative movement of position 52a involves a change in the

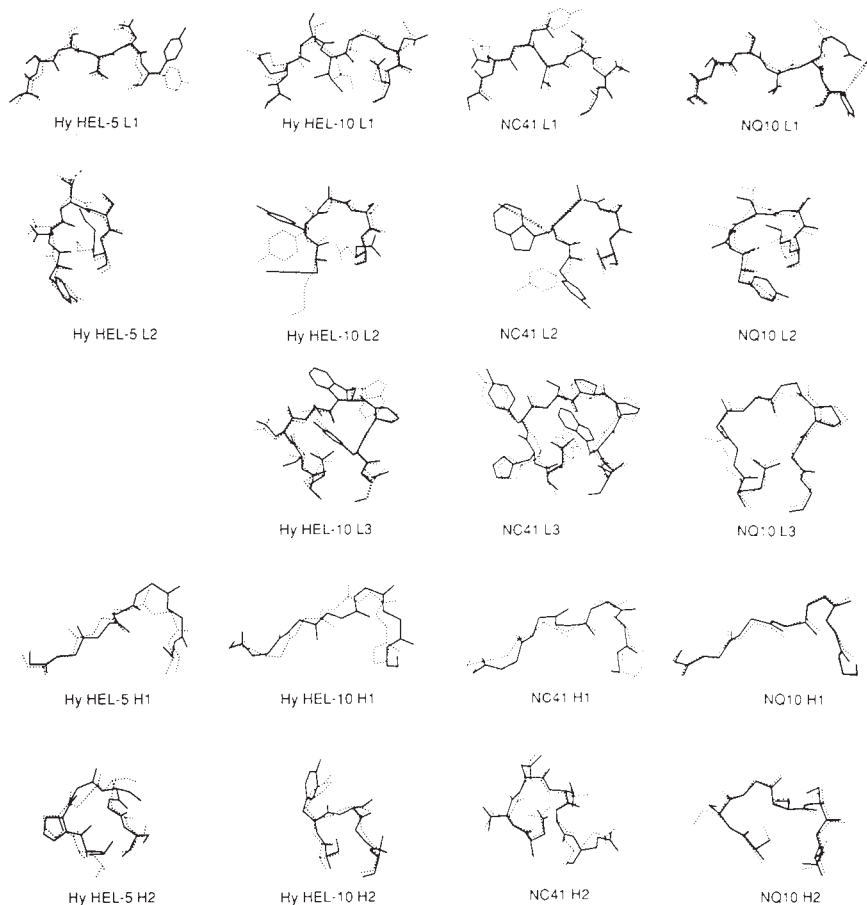


FIG. 2 The predicted (broken line) and observed (continuous line) conformations of the hypervariable regions. The structures have been superposed by a least-squares fit of their main-chain atoms. Residue numbers and the r.m.s. difference in position of the superposed atoms are given in Table 2a. Predicted and observed side-chain conformations are shown for all regions except H1 and L3 in NQ10 where they obscure the main chain. After our prediction of the NC41 structure several revisions were made to the sequence and some of the differences can be seen here.

TABLE 2 Differences in structure of predicted and observed hypervariable regions

(a) Differences in local conformation (Å)				
Hypervariable region	R.m.s. difference in atomic positions of main-chain atoms after optimal superposition			
	HyHEL-5	HyHEL-10	NC41	NQ10
L1:26-32	0.8	1.1	0.7	0.4
L2:49-53	0.9	0.8	0.4	0.9
L3:90-97	—	0.3	0.5	0.6
H1:26-32	1.4	1.3	0.9	0.3
H2:52-56	1.1	1.0	0.7	0.4

(b) Differences in position relative to framework (Å)			
Range of differences in positions of C _α atoms after superposition of framework residues (Å)			
Protein	Hypervariable region	Original prediction	New structure library
HyHEL-5	L1	2.0-3.8	0.8-2.1
	L2	1.6-1.6	1.2-2.3
	H1	0.8-4.1	0.7-2.1
	H2	3.0-7.2	0.5-2.1
HyHEL-10	L1	0.7-1.6	—
	L2	0.6-1.3	—
	L3	0.8-1.5	—
	H1	1.3-3.5	—
NC41	L1	1.4-2.4	1.5-2.6
	L2	1.1-1.8	1.0-2.0
	L3	1.6-2.3	1.8-3.0
	H1	1.3-2.0	1.0-2.1
NQ10	H2	2.1-4.4	0.4-1.9
	L1	0.4-2.7	—
	L2	0.5-1.4	—
	L3	0.6-1.5	—
	H1	0.6-1.2	—
	H2	0.6-0.9	—

Superposition of *a* are illustrated in Fig. 2. The differences in the positions of the hypervariable regions in the original predictions and the observed structures of *b* are shown in Fig. 3. The predictions with a new structure library involve rebuilding the HyHEL-5 model using the refined VL J539 and the VH NC41 structures, and rebuilding the NC41 model using VL McPC603 and VH HyHEL-5; see text.

conformation of H2. It also tilts the H2 loop so that the positions of residues at the top of the loop in HyHEL-5 differ in position, relative to those in KOL and J539, by ~4.5 Å. In NC41, the residue at position 71 is Leu and that at position 52a is Thr, and the shift is not as large as that in HyHEL-5.

This analysis implies that the conformation and position of four residue H2 regions are determined by the packing against the VH framework and by the identity of the residue at position 71 in particular, and not by the position in the sequence of H2 of Gly (or Asn or Asp) as was believed previously⁸. Thus VH domains of HyHEL-5 and NC41 should provide better parents for each other than the other structures do, for they contain similar determinants for the position of H2. The predicted structure of HyHEL-5 using the observed structure of VH of NC41 and the predicted structure of NC41 using the observed structure of VH of HyHEL-5 were thus rebuilt. In these new predicted structures the large differences between the predicted and observed positions of H2 are not present: most residues differ by no more than 2.1 Å and none differs by more than 3.0 Å (Table 2b).

The results of this analysis were used in the prediction of the structure of the H2 region of the antibody NQ10.

Hypervariable regions of NQ10 and D1.3

The amino-acid sequence of the hypervariable regions and associated framework sites of NQ10 are given in Table 1. For five of the hypervariable regions, the size and the residue conservation at the relevant sites clearly indicate particular canonical structures, and a model of the VL-VH dimer of NQ10 was made using the procedure described above. For H2, after the

analysis described above, canonical structure 2 was expected because of the Arg at position 71 (Table 1).

Recently a crystal structure has been determined for NQ10 (S.S., P.M.A. and R.J.P., manuscript in preparation). This structure is determined to a resolution of 2.8 Å and the present residual is 21%. The tracing of the chain in the hypervariable regions is unambiguous. Figure 2 shows the predicted and observed structures of each of the hypervariable regions, superposed by a least-squares fit of their main-chain atoms. Table 2a gives the r.m.s. differences in position of the main-chain atoms. Figure 3 shows the relative positions of the predicted and observed hypervariable regions.

There is close agreement between the predicted and observed main-chain conformations of the hypervariable regions: the r.m.s. differences in position are between 0.3 and 0.9 Å (Table 2a). There is also close agreement in the relative positions of the hypervariable regions (Fig. 3 and Table 2b). No residues differ by more than 2.7 Å in position and all but two are within 1.7 Å.

Recently, the atomic structure of antibody D1.3 complexed with lysozyme has been refined²⁰. The comparison of the preliminary experimental structure with the prediction had shown differences for two hypervariable regions⁹. The L3 regions had differences associated with Pro 95 having a *cis*-peptide in the predicted structure and a *trans*-peptide in the observed. The H1 regions differed because the predicted structure had this region folded into framework, whereas in the observed it folded out into the solvent. The refinement of the experimental structure has resulted in the rebuilding of these two regions²⁰, and their conformations now agree with the original predictions.

Canonical structures in immunoglobulin 4-4-20

The atomic structure of immunoglobulin 4-4-20 has recently been determined at a resolution of 2.7 Å (ref. 19). The amino-acid sequence of the hypervariable regions and associated framework sites of 4-4-20 are given in Table 1. Four of the hypervariable regions, L2, L3, H1 and H2, have the size and the residues at specific sites that clearly indicate particular canonical structures (see Table 1). These four hypervariable regions do have the expected main-chain conformations. In Fig. 4 the four hypervariable regions, superposed on examples of the same canonical structures taken from other immunoglobulins, are shown. The r.m.s. difference in the atomic

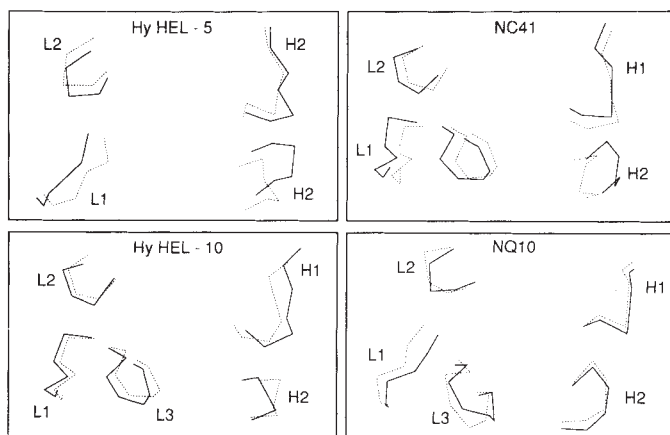


FIG. 3 The relative positions of the hypervariable regions in the predicted and observed structures. The positions of the hypervariable regions shown are those given by the superposition of the VL-VH framework regions of the observed and predicted structures. The C_α atoms of residues in the observed structure are joined by solid lines and those in the predicted structure by broken lines. The size of the differences in position are given in Table 2b.

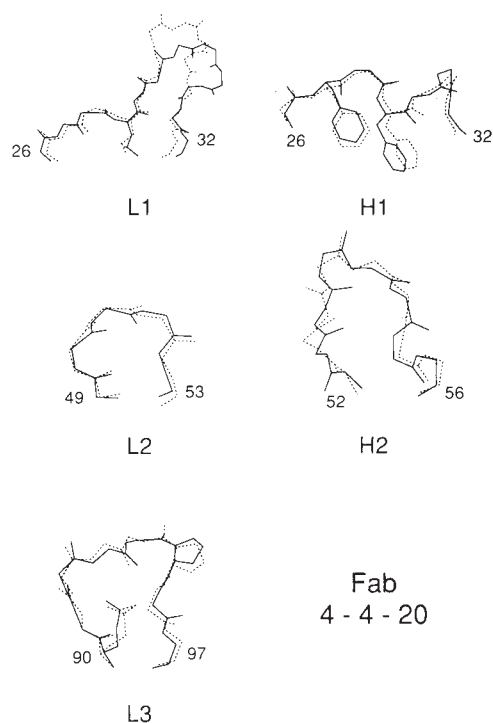


FIG. 4 The hypervariable regions in immunoglobulin 4-4-20. The observed conformations of the hypervariable regions in 4-4-20 are drawn with continuous lines. They are superposed on hypervariable regions from other immunoglobulins that have the same canonical structure—L2 and L3 from REI, H1 from KOL and H2 from McPC603—or in the case of L1, the closely related canonical structure from McPC603. The side chains shown are those that are important in determining the conformation of the region.

position of the main-chain atoms of these pairs are in the range 0.5–0.9 Å.

The L1 region of 4-4-20 has a different size to those found previously and represents a new canonical structure (see Table 1 and Fig. 4). As predicted in a previous discussion of the sequence patterns of this region⁸, its conformation is closely related to those of the other canonical structures known for this region. The main-chain atoms of residues 26–31 and 31e–32 fit the same region in McPC603 with a r.m.s. difference in position of 0.8 Å (Fig. 4).

Common occurrence of known canonical structures

The close agreement of the predicted and observed main-chain conformations of the hypervariable regions strongly supports

the canonical structure model. It implies that a good estimate can be made of the extent to which hypervariable regions in other immunoglobulins have main-chain conformations close to those that are now known. This can be done by inspecting the known sequences to see whether their hypervariable regions correspond in size to the known canonical structures, and whether they contain one of the sets of residues that produce the observed conformations.

In Table 1 we give the results of examining the immunoglobulin sequences collected by Kabat *et al.*²⁹. About 90% of the hypervariable regions in V_K domains, and ~70% of the H1 and H2 regions in V_H domains, are expected to have conformations close to those found in the immunoglobulin structures now known. These estimates are conservative in that they include only hypervariable regions that match the sequence requirements exactly, that is, those that contain the particular residues, or any combination of the small range of closely related residues, that are found in the known structures at the sites marked by asterisks in Table 1.

Discussion

The wide occurrence of the known canonical structures, the use of the much larger library of parent structures that will soon become available, and the more detailed understanding of the determinants of structure that will emerge from the analysis of the differences between predicted and observed structures, should allow, for many immunoglobulins, the prediction of the structure of five of the hypervariable regions with accurate local conformation and errors in position of 2 Å or less.

Predictions of the structures of the H3 regions in the immunoglobulins discussed here could not be made on the basis of the previously known structures. It was possible to predict correctly the H3 conformation in immunoglobulin D1.3 (ref. 9). In general, however, the various genetic mechanisms that produce the H3 regions usually result in medium or large surface loops with very different sequences and patterns of interaction. For such hypervariable regions it may be possible to predict their structure using the conformational search algorithms that have been developed by several groups^{30–35}.

The results presented here have interesting implications for the molecular mechanisms involved in the generation of antibody diversity. For at least five of the six hypervariable regions of most immunoglobulins there seems to be only a small repertoire of main chain conformations, most of which are known from the set of immunoglobulin structures so far determined. Sequence variations within the hypervariable region modulate the surface that these canonical structures present to the antigens. Sequence variations within the framework and hypervariable regions shift the canonical structures relative to each other by small but significant amounts. □

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Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP

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In vitro reconstitution of active ribulose biphosphate carboxylase (Rubisco) from unfolded polypeptides is facilitated by the molecular chaperones: chaperonin-60 from *Escherichia coli* (groEL), yeast mitochondria (hsp60) or chloroplasts (Rubisco subunit-binding protein), together with chaperonin-10 from *E. coli* (groES), and Mg-ATP. Because chaperonins are ubiquitous, a conserved Mg-ATP-dependent mechanism exists that uses the chaperonins to facilitate the folding of some other proteins.

THE chaperonins¹ are a ubiquitous class of proteins implicated in the post-translational folding and assembly of other proteins. They include the groEL or chaperonin-60 (cpn60) and the groES or chaperonin-10 (cpn10) proteins from bacteria²⁻⁴, chloroplasts⁵ and mitochondria⁶⁻⁸. The earliest evidence for the involvement of the chaperonins with post-translational events was the demonstration that the *E. coli* cpn60 and cpn10 proteins are essential for phage head morphogenesis⁹⁻¹¹. The chloroplast version of cpn60 is the Rubisco subunit-binding protein, so-called because of its transient association with newly synthesized Rubisco subunits¹²⁻¹⁴. The transient association of newly synthesized unfolded proteins with the chaperonins has also been observed during the formation of pre-beta-lactamase and chloramphenicol acetyltransferase¹⁵ in *E. coli*, during the recombinant expression in *E. coli* of the precursor to the small subunit of Rubisco¹⁶, and also after the import of precursor proteins into yeast mitochondria^{17,18} and chloroplasts¹⁹. But the molecular details of what happens while these polypeptides are associated with the chaperonins remains to be determined.

Recently, we demonstrated *in vivo* that the *E. coli* chaperonins cpn60 and cpn10 promoted the post-translational folding or assembly, or both, of hexadecameric (L₈S₈) and dimeric (L₂) Rubiscos in *E. coli*²⁰. We suggested that the chaperonins exert their effect post-translationally, at some point between the unfolded nascent monomer (L) and the formation of the folded dimer (L₂), the basic structural unit of the more complex hexadecamer²¹⁻²³. We have now used the purified *E. coli* chaperonins cpn60 and cpn10 to demonstrate that the *in vitro* reconstitution of the catalytically functional dimeric form of Rubisco from an unfolded biologically inert polypeptide depends on the presence of both chaperonin proteins and Mg-ATP.

The substrate—denatured Rubisco

Rubisco from *Rhodospirillum rubrum* is a dimer of large subunits only²⁴. Genetic²⁵ and structural²¹ studies have established that the dimer is the minimal structural entity capable of catalysis, a single active site being composed of amino-acid residues from both subunits.

Because our genetic analysis²⁰ indicated that the chaperonins functioned post-translationally at some point before the formation of the folded functional dimer, we sought conditions for unfolding native Rubisco in the belief that unfolded Rubisco would serve as a substrate for the chaperonins. To determine the change in secondary structure accompanying the various denaturing regimes, we recorded circular dichroism (CD) spectra in the far ultraviolet range. The spectrum for native decarbamylated Rubisco (Fig. 1, E) showed negative molar ellipticity ($\theta_{220} = -10^4$ degrees·cm²·dmol⁻¹) that is expected for a protein with this structure. On denaturation with either 8 M

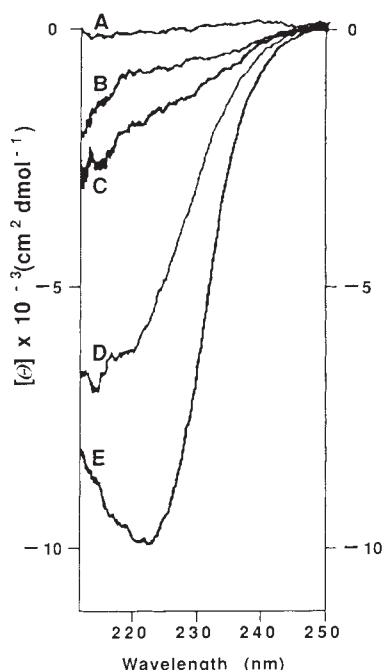


FIG. 1 CD spectra of native and denatured Rubiscos. A, baseline; B, denatured Rubisco (Rubisco-U) in 6 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.85; C, denatured Rubisco (Rubisco-U) in 8 M urea, 0.1 M Tris-HCl buffer, pH 7.85; D, denatured Rubisco (Rubisco-A) in 0.1 M glycine-HCl, pH 3.0; and E, native decarbamylated Rubisco in 0.1 M Tris-HCl buffer, pH 7.85. Concentration of Rubisco protomer, 7.9 μM; temperature, 25 °C.

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urea (Fig. 1, C) or 6 M guanidine-HCl (Fig. 1, B), the negative ellipticity was strongly reduced indicating a considerable loss of secondary structure. We conclude that treatment with either 8 M urea or 6 M guanidine-HCl converts Rubisco into unfolded monomeric polypeptides. We refer to Rubisco in this state as Rubisco-U.

Treatment of Rubisco with acid (pH 3) led to a rapid ($t_{1/2} \sim 30$ s) loss of activity which was not recovered on neutralization. The CD spectrum of acid-denatured Rubisco (Fig. 1, D) showed some reduction of the negative band ($\theta_{220} = -6 \times 10^3$ degrees·cm²·dmol⁻¹). It is evident, however, that much secondary structure remains under these conditions. Whether or not the structure of the acid-denatured Rubisco corresponds to the molten-globule or A-state, observed with other proteins²⁶⁻²⁸ at acidic pH, requires further investigation. We refer to acid-denatured Rubisco in this partly unfolded state as Rubisco-A.

Chaperonin-dependent Rubisco reconstitution

Attempts to spontaneously reconstitute active Rubisco from both Rubisco-U and Rubisco-A under various conditions were unsuccessful. By contrast, when either Rubisco-U or Rubisco-A was diluted into solutions containing both chaperonins cpn60 and cpn10, and Mg-ATP, Rubisco activity, indicative of a folded dimer, was reconstituted (Fig. 2a). No reconstitution occurred when cpn60 or cpn10 was omitted. The time course of reconstitution was followed by using a glucose-hexokinase quench, demonstrating the requirement for Mg-ATP (Fig. 2a, b). The maximum reconstitution was $\sim 80\%$. Rubisco-U consistently yielded the greatest quantity of reconstituted enzyme. Rubisco-A was reconstituted to $\sim 40\%$ (Fig. 2a). Although the extent of reconstitution varied, depending on whether Rubisco-U or Rubisco-A was used as substrate, the rate constant for the chaperonin-dependent reconstitution was the same, within the limit of experimental error. This implies that reconstitution

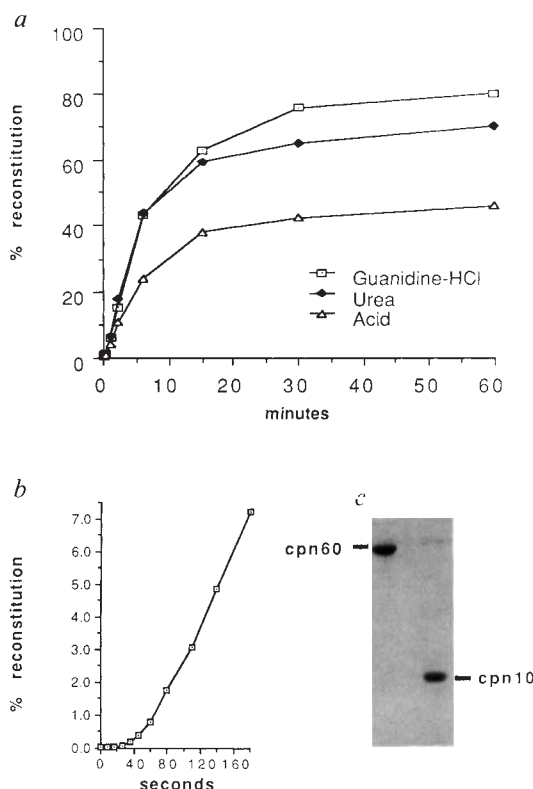
involves a common intermediate, the formation of which precedes the same chaperonin-dependent rate-determining step. We propose that on removal of the denaturant by dilution, a rapid chaperonin-independent folding of Rubisco-U to a state rich in secondary structure and resembling Rubisco-A (Rubisco-I state), is followed by reaction with cpn60, cpn10 and Mg-ATP.

Reconstitution of Rubisco involves two processes. Folding of the polypeptide, which is independent of concentration, followed by the assembly of the folded but inactive monomers into catalytically competent dimers. Assembly depends on the concentration of monomers and on the dissociation constant governing their interaction. The magnitude of this dissociation constant is so far unknown. We anticipated that the appearance of Rubisco activity might, however, be preceded by a lag period, during which sufficient folded monomers accumulate before dimerization. Inspection of the earliest part of the reconstitution (Fig. 2b) revealed just such a lag. We estimate that detectable dimer formation requires the accumulation of ~ 2 nM folded monomers. A similar value was computed after the synthesis of Rubisco in a prokaryotic cell-free transcription-translation system²⁹.

The chaperonin-dependent reconstitution system was examined as a function of the concentrations of Rubisco, cpn60 and cpn10, varying the concentration of one component at a time (Fig. 3). First, no reconstitution occurred when either cpn10 (Fig. 3a) or cpn60 (Fig. 3b) was omitted. Second, optimal reconstitution occurred when cpn60 and cpn10 were approximately equimolar (Fig. 3a, b). Note, however, that reconstitution was inhibited when the concentration of cpn60 exceeded that of cpn10 (Fig. 3b).

The dependency of the reconstitution system on the Rubisco concentration was more complex (Fig. 3c) and reflected the interplay of two processes. At the lowest concentration of Rubisco used (3 nM protomer), only 20% reconstitution occurred. But the yield of active enzyme increased with increas-

FIG. 2 The time-course of chaperonin- and Mg-ATP-dependent reconstitution of active Rubisco from variously denatured Rubiscos (Rubisco-U or Rubisco-A). a, Complete time courses of reconstitution. b, Lag in the formation of active Rubisco dimers from a different experiment to that represented in a. In both cases Rubisco activity is expressed as a percentage of the activity of an equal quantity of native non-denatured enzyme. c, SDS-PAGE of the purified preparations of cpn60 and cpn10 from *E. coli* used in this study. METHODS. The *E. coli* chaperonins cpn60 and cpn10 were purified from lysates of cells bearing the multicopy plasmid pGroESL²⁰, by modifications (to be described elsewhere) of published purification protocols^{30,38}. Recombinant dimeric Rubisco was purified from *E. coli* expressing a plasmid-encoded gene from *R. rubrum* as previously described^{39,40}. a, Rubisco-U was prepared by incubating 15.4 μ M Rubisco (protomer) in 0.1 M Tris-HCl buffer, 0.01 M EDTA, pH 7.7, containing either 5.7 M guanidine-HCl or 7.6 M urea for 2 h at 25 °C. Rubisco-A was prepared by incubating 15.4 μ M Rubisco (protomer) in 50 mM glycine-HCl, pH 3.0, for 210 s at 25 °C. Reconstitution was initiated by diluting 6.7 μ l of the denatured Rubisco-U or Rubisco-A into 1.418 μ l solution containing 50 mM Tris-HCl buffer, pH 7.5, 2 mM ATP, 12 mM MgCl₂, 10 mM glucose, 4.3 μ M cpn60 (protomer) and 9.6 μ M cpn10 (protomer) at 25 °C. The final concentration of Rubisco protomer in the reconstitution reaction was 72 nM. At the times indicated a 150 μ l aliquot was withdrawn and added to 10 μ l solution containing 6 U of hexokinase so as to arrest the reconstitution reaction. After the last aliquot had been withdrawn, the reaction mixtures were supplemented with 50 mM [¹⁴C]NaHCO₃ (300 d.p.m. nmol⁻¹) and allowed to activate for a further 5 min. Ribulose biphosphate (1 mM) was added and Rubisco activity measured as the formation of acid-stable ¹⁴C over 30 min at 25 °C. The activity of native Rubisco (100%) under these conditions was 2.7 s⁻¹. b, Rubisco-U (20.4 μ M protomer) was prepared in guanidine-HCl as described above. Reconstitution was initiated by diluting 21 μ l Rubisco-U into 1.659 μ l solution containing 50 mM Tris-HCl buffer, pH 8.0, 3 mM ATP, 7 mM MgCl₂, 20 mM glucose, 4 μ M cpn60 (protomer) and 6 μ M cpn10 (protomer) at 23 °C. At the times indicated, the reconstitution reaction was quenched with hexokinase and Rubisco activity determined as described above. c, Samples of purified cpn60 and cpn10 containing ~ 4 μ g were boiled in 1% SDS for 5 min and applied to a 16% SDS-polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue.



ing concentration until an optimum (for this particular concentration of chaperonins) was reached at ~ 50 nM Rubisco. These results are again consistent with the need to accumulate sufficient folded monomers to drive the assembly of active dimers. Optimal reconstitution was reached at a cpn60: Rubisco-protomer concentration ratio of $\sim 50:1$ (Fig. 3c). But because reconstitution involved the oligomeric form of cpn60, the double-doughnut-shaped 14-mer^{30,31} ([cpn60]₁₄) (Fig. 4, B and C), the molar ratio of [cpn60]₁₄ to Rubisco protomers was closer to 3:1. The requirement for excess cpn60 over Rubisco was largely due to the instability of the partly folded intermediate, Rubisco-I, that is believed to be generated from both Rubisco-U and Rubisco-A on removal of the denaturant, and its propensity to irreversibly aggregate (Fig. 4). The decline in the yield of reconstituted Rubisco observed as the concentration of Rubisco-U was increased was due to irreversible aggregation (Fig. 3c). Thus, when either Rubisco-U or Rubisco-A was diluted into solutions containing the chaperonins, two competitive and mutually exclusive processes occurred. The unfolded or partly folded protomers either formed biologically unproductive aggregates or they formed a binary complex with [cpn60]₁₄ (see below), which prevented aggregation and directed the protomers along a biologically productive pathway.

Homologous cpn60s also support reconstitution

The prokaryotic cpn60 is homologous to the eukaryotic chaperonins hsp60, from yeast mitochondria, and the Rubisco subunit-binding protein, from plant chloroplasts^{5,7}. We purified both proteins to homogeneity and used them in a heterologous reconstitution system with *E. coli* cpn10. We used approximately equal concentrations of each of the cpn60 proteins together with equal concentrations of cpn10. The heterologous chaperonin combinations cpn60 (yeast)-cpn10 (*E. coli*), and cpn60 (chloroplast)-cpn10 (*E. coli*) were respectively 10% and 25% as effective as the homologous cpn60-cpn10 (*E. coli*) combination in facilitating the reconstitution of Rubisco-U. This was not unexpected, because the chaperonins of a given organism have presumably evolved to function together.

Cpn60 traps an unstable folding intermediate

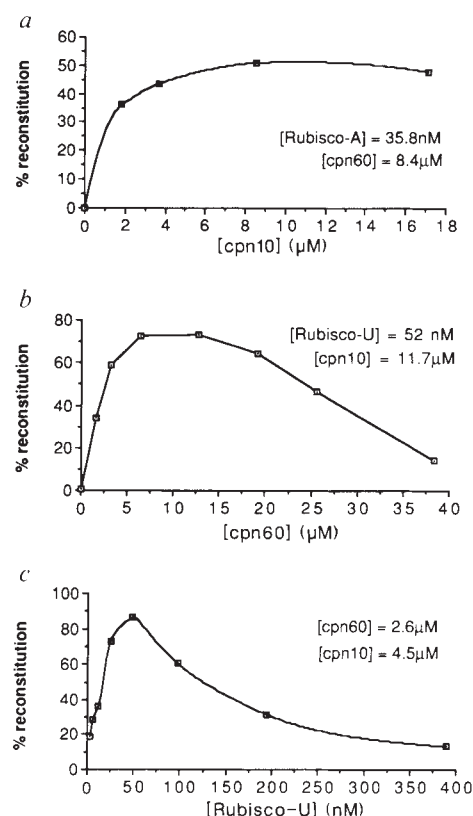
Evidence that Rubisco-U is rapidly converted to an unstable intermediate state, Rubisco-I, on removal of the denaturant, and the ability of cpn60 to stabilize this intermediate was provided by the delay experiment, the results of which are shown in Fig. 4. In this experiment, Rubisco-U was diluted into a solution lacking cpn60. Cpn60 was added 5–60 s later. Cpn10 was added ~ 10 min later to complete the reconstitution mixture. The ability of the chaperonins to reconstitute activity from Rubisco-U was rapidly lost as the time between the dilution of Rubisco-U and the addition of cpn60 increased. The rate at which recoverable activity was lost increased as the concentration of Rubisco protomers increased (Fig. 4), indicating that an unstable folding intermediate—Rubisco-I, formed from Rubisco-U on dilution—was susceptible to irreversible aggregation. This accounts for the progressive decline in the yield of reconstituted Rubisco observed as the concentration of Rubisco-U introduced into the system was increased (Fig. 3c). We conclude that when Rubisco-U is diluted into solutions containing the chaperonins, two competitive and mutually exclusive processes occur—aggregation or formation of a binary complex with [cpn60]₁₄.

Rubisco-I-[cpn60]₁₄ binary complex is stable

We demonstrated the physical existence of a stable binary complex between Rubisco-I and cpn60, and the conditions leading to the formation of an active dimer of Rubisco, by immunoelectrophoretic procedures (Fig. 5). When Rubisco-U was diluted into a solution containing cpn60, cpn10 and Mg-ATP, Rubisco activity, indicative of folded dimers, was reconstituted (Fig. 5a, column 7). The dimer was detected immunochemically after non-denaturing PAGE (Fig. 5b, track 7). When one or more of the components was omitted, or when the chaperonins were first boiled, no reconstitution of Rubisco activity occurred (Fig. 5a, columns 2–6, 8 and 9, respectively) and no cross-reactive band corresponding to the dimer was detected (Fig. 5b, tracks 2–6, and 8 and 9, respectively). In the presence of native cpn60, but when either cpn10 or Mg-ATP or both were absent,

FIG. 3 The chaperonin- and Mg-ATP-dependent reconstitution of active Rubisco as a function of the concentrations of Rubisco, cpn60 and cpn10. The yield of reconstituted Rubisco as function of variable cpn10 concentration, as indicated, constant cpn60 concentration, constant Rubisco concentration (a); variable cpn60 concentration as indicated, constant cpn10 concentration, constant Rubisco concentration (b); variable Rubisco concentration as indicated, constant cpn60 concentration, constant cpn10 concentration (c). Note that these are not measurements of the initial rates of reconstitution, but the extent of complete reconstitution after 1–3 h.

METHODS. a, Rubisco-A was prepared by incubating 1.8 mM Rubisco (protomer) in 50 mM glycine-HCl, pH 3.0, for 4 min at 23 °C. Reconstitution was initiated by diluting 5 μ l Rubisco-A into 245 μ l solution containing 75 mM Tris-HCl buffer, pH 7.7, 7.5 mM MgCl₂, 5 mM ATP, 1 mg ml⁻¹ BSA, 8.4 μ M cpn60 (protomer) and various concentrations (0–17.8 μ M protomer) of cpn10. A control containing an equal quantity of native Rubisco was also prepared. After incubation for 1 h at 23 °C, Rubisco activity was determined as described in the legend to Fig. 2. b, Rubisco-U (3.9 μ M protomer) was prepared as described above. Reconstitution was initiated by diluting 4 μ l Rubisco-U (3.9 μ M) into 296 μ l solution containing 11.7 μ M cpn10 (protomer), 50 mM Tris-HCl buffer, pH 8.0, 15 mM MgCl₂, 4 mM ATP and various concentrations (0–38.4 μ M protomer) of cpn60. Controls containing an equal quantity of native Rubisco were prepared. After incubation at 25 °C for 1 h, Rubisco activity was determined as described in the legend to Fig. 2. c, Various concentrations (0.31 to 39 μ M protomer) of denatured Rubisco-U were prepared by incubating the appropriate amount of Rubisco in 5.7 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.7, 0.01 M EDTA for 1 h at 23 °C. Reconstitution was initiated by diluting 2.5 μ l Rubisco-U into 247.5 μ l solution containing 2.6 μ M cpn60 (protomer), 4.5 μ M cpn10 (protomer), 2 mM ATP, 12.5 mM MgCl₂ and 75 mM Tris-HCl buffer, pH 7.8. Controls using the same quantity of native Rubisco were prepared. After incubation for 3 h at 25 °C, Rubisco activity was determined as in the legend to Fig. 2, with the exception that the duration of the Rubisco assay was varied according to the quantity of enzyme present.



two electrophoretically distinct immunochemically cross-reactive signals were observed (Fig. 5b, tracks 3, 4 and 6). The first sharply defined band, revealed with antibody against Rubisco, corresponded to the binary complex of Rubisco subunits bound to the oligomeric form $[\text{cpn60}]_{14}$. The same oligomer of cpn60, whether or not associated with Rubisco-I, was also revealed with antibody against cpn60 (Fig. 5c, tracks 1, 3, 4, 6 and 7).

The second cross-reactive signal revealed with antibody against Rubisco, represented a poorly defined smear of Rubisco subunits with a slower electrophoretic mobility than the dimer (Fig. 5b, tracks 3, 4 and 6). The nature of these soluble Rubisco subunits is puzzling. They have no catalytic activity. Perhaps they are misfolded or partly folded monomers which arise during electrophoresis by dissociation of the binary complex.

The binary complex could also be demonstrated starting with acid-denatured Rubisco-A (data not shown). Therefore, we conclude that a common folding intermediate, Rubisco-I, is generated from both Rubisco-U and Rubisco-A on dilution, and that Rubisco-I is stabilized by forming a binary complex with $[\text{cpn60}]_{14}$. Because Rubisco-A, and by inference Rubisco-I, has significant secondary structure (Fig. 1), it follows that considerable folding of Rubisco-U to the state represented by Rubisco-I must occur before interaction with cpn60.

The experiment represented in Fig. 5 provided further insight into the mechanism of the chaperonin-dependent reconstitution reaction. First, note that native Rubisco showed no detectable interaction with cpn60 (Fig. 5b, c; tracks 1 and 10). In the absence of Mg-ATP, cpn60 moved electrophoretically on non-denaturing gels as a single oligomeric species of high relative molecular mass, $[\text{cpn60}]_{14}$ (refs 30 and 32). In the presence of Mg-ATP, however, dissociation of $[\text{cpn60}]_{14}$ to a species of lower relative molecular mass occurred, and a second band, designated $[\text{cpn60}]_n$, appeared on the western blot (Fig. 5c, tracks 1, 4, and 7–9). The number of cpn60 subunits in $[\text{cpn60}]_n$ is not known. The chloroplast cpn60, the Rubisco subunit-binding protein, has been reported to behave similarly^{13,32}. Although the addition of Mg-ATP to the mixture of the binary

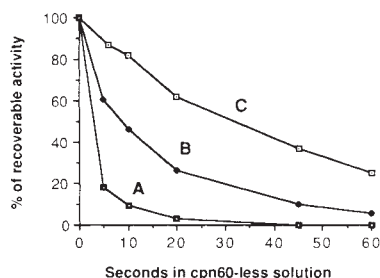


FIG. 4 The instability of the folding intermediate $[\text{Rubisco-I}]_1$ in the absence of cpn60 and the concentration dependence of its decay-aggregation. Rubisco-U 109 nM protomer (A), 54.3 nM protomer (B) or 27.2 nM protomer (C) was incubated in solutions not containing cpn60 (cpn60-less solutions) for the indicated times, when cpn60 was added to stabilize the folding intermediate $[\text{Rubisco-I}]_1$.

METHODS. Rubisco-U at three different concentrations (10.9 μM , 5.4 μM and 2.7 μM protomer) was prepared in 5.7 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.7, 1 mM dithiothreitol and 0.1 mM EDTA. At time zero, 50- μl aliquots of Rubisco-U were diluted into 950 μl solution containing 50 mM Tris-HCl buffer, pH 8.0, 0.5 mg ml^{-1} BSA, 15 mM MgCl_2 , and 5 mM ATP at 23 °C. At the times indicated, 50- μl aliquots were withdrawn and added to 250 μl solution containing 50 mM Tris-HCl buffer, pH 8.0, 15 mM MgCl_2 , 5 mM ATP and 6.3 μM cpn60 (protomer) so as to give the following concentrations of Rubisco protomer: 109 nM (A), 54.3 nM (B) and 27.2 nM (C). About 10 min after the last aliquot had been withdrawn, 6 μl cpn10 (protomer; 145 μM) were added to initiate reconstitution. After 1 h at 25 °C, Rubisco activity was determined as described in the legend to Fig. 2. For the time-zero points, 2.5 μl Rubisco-U were diluted directly into 297.5 μl cpn10-less reconstitution mixture. Subsequent treatment was as described above. Controls using equal quantities of native Rubisco were prepared. The data are normalized to the time-zero points for each concentration of Rubisco-U.

complex and uncomplexed $[\text{cpn60}]_{14}$ caused the formation of a smaller species of cpn60, Mg-ATP alone did not lead to the formation of active Rubisco (Fig. 5a, column 4), nor to the discharge of the binary complex (Fig. 5b, track 4). Similarly, the addition of cpn10 alone led neither to the formation of active Rubisco (Fig. 5a, column 6) nor to the discharge of the binary complex (Fig. 5b, track 6). Only when both cpn10 and Mg-ATP were added was the binary complex discharged with the

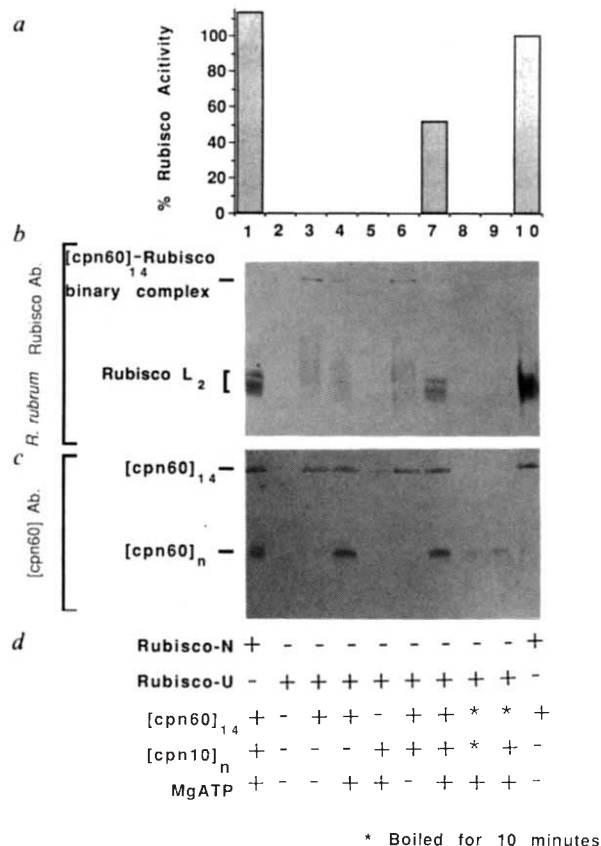


FIG. 5 Chaperonin- and Mg-ATP-dependent reconstitution of active dimeric Rubisco from denatured Rubisco-U. a, Rubisco activity, expressed as a percentage of the activity observed with an equal quantity of non-denatured, native Rubisco. b, Western blot after non-denaturing PAGE of the reaction mixtures used in a and probed with antibody (Ab) raised against Rubisco. The positions of $[\text{cpn60}]_{14}$ and of native Rubisco are indicated. The binary complex of $[\text{cpn60}]_{14}$ -Rubisco was not resolved from uncomplexed $[\text{cpn60}]_{14}$. Note the association of Rubisco with $[\text{cpn60}]_{14}$ in tracks 3, 4 and 6. The achromatic band in the middle of the native Rubisco bands in tracks 1 and 7 is due to cpn10, which interferes with the interaction of Rubisco and its antibody. c, As in b, but probed with antibody raised against cpn60. Note the dissociation of $[\text{cpn60}]_{14}$ to a lower relative molecular mass species, $[\text{cpn60}]_n$, in the presence of Mg-ATP. d, Summary of the reaction conditions.

METHODS. Denatured Rubisco-U was prepared by incubating 15.4 μM Rubisco (protomer) in 5.7 M guanidine-HCl, 0.1 M Tris-HCl buffer, pH 7.7, 1 mM dithiothreitol and 0.1 mM EDTA for 3 h at 25 °C. Reconstitution was initiated by diluting 2.5 μl above solution into 247.5 μl solution containing (when complete) 50 mM Tris-HCl buffer, pH 7.7, 4.0 mM ATP, 12 mM MgCl_2 , 1 mM dithiothreitol, 4.0 μM cpn60 (protomer) and 7.6 μM cpn10 (protomer). Individual components were omitted as indicated in d. When chaperonins were omitted (as in tracks 2–5 and 10), an equivalent molar concentration of BSA was added. Boiled (100 °C, 10 min) chaperonins were added as indicated (tracks 8 and 9). Controls employing an equal concentration of native Rubisco (tracks 1 and 10) were prepared. Alternatively, after the period for reconstitution, aliquots of the reaction mixtures were analysed by non-denaturing PAGE on 6.5% gels, followed by immunodetection using antibodies raised against either Rubisco (b) or cpn60 (c). To suppress the nonspecific binding of anti-Rubisco antibodies to cpn60 in b, 15 $\mu\text{g}/\text{ml}^{-1}$ purified cpn60 was added to the 5% milk-blocking mixture.

appearance of the native dimer (Fig. 5*b*, track 7) and of Rubisco activity (Fig. 5*a*, column 7). From these experiments we conclude that the chaperonin-dependent reconstitution of Rubisco involves a strictly ordered set of reactions—first, the formation of a stable binary complex of [cpn60]₁₄–[Rubisco-I]₁, which at least *in vitro* can be demonstrated as an Mg-ATP- and cpn10-independent event, followed by the Mg-ATP- and cpn10-dependent discharge of folded and stable, but catalytically inactive, monomers, which subsequently assemble into active dimers.

Cpn10 and ATP react with the binary complex

We determined the initial rate of reconstitution as a function of the concentration of Mg-ATP. On this basis the Michaelis constant K_m for Mg-ATP was ~0.3 mM (data not shown). Other nucleotide triphosphates (CTP, GTP, UTP and dATP) were investigated for their ability to support reconstitution. Rubisco activity was determined after one hour's incubation in the presence of cpn60, cpn10 and 5 mM nucleotide triphosphate. No reconstitution was seen with GTP, UTP and dATP. But CTP supported a chaperonin-dependent reconstitution of Rubisco activity, with a yield of ~50%. Non-hydrolysable analogues of ATP (α,β -methylene adenosine 5'-triphosphate, β,γ -methylene adenosine 5'-triphosphate and β,γ -imidoadenosine-5'-triphosphate) did not support reconstitution. Nor did adenosine 5'-(γ -thio)triphosphate support reconstitution. Furthermore, adenosine 5'-(γ -thio) triphosphate inhibited the ATP-dependent reconstitution reaction (data not shown), indicating that at least this ATP-analogue can bind to the chaperonin protein. The failure of these ATP analogues to support the chaperonin-dependent reconstitution of Rubisco indicates that the hydrolysis of ATP could be involved.

Discussion

The essential function of the chaperone proteins, as proposed by Pelham³³, and by Ellis and colleagues^{1,34}, is to facilitate the folding of other polypeptides and their assembly into oligomeric structures of which they are not a part. It was further proposed that molecular chaperones both prevent the formation of incorrect structures and unscramble those that do occur. The experiments reported here provide experimental evidence both for and against the three elements of this hypothesis. First, the *E. coli* chaperonins cpn60 and cpn10 facilitate a Mg-ATP-dependent conversion of an unfolded or partly folded polypeptide—Rubisco in this instance—into a correctly folded catalytically active dimer, in accordance with the main postulate. Second, the folding of Rubisco involves an unstable intermediate, Rubisco-I, with a propensity to aggregate to form biologically unproductive structures. By forming a stable binary complex with cpn60, the formation of these incorrect structures is prevented and the folding intermediate directed down a biologically productive pathway. But the chaperonins are unable to rescue Rubisco molecules which aggregate. This step seems irreversible, at least on the timescale of our experiments.

The failure of both Rubisco-U and Rubisco-A to fold spontaneously under the conditions we have used and within the time frame of the experiments described here does not exclude the possibility that they could be induced to do so under a different set of conditions. But regardless of that, we are left with the fact that the chaperonins enhance the rate of reconstitution of Rubisco under the chosen conditions by a very substantial factor. Therefore, along with *cis-trans* prolyl isomerase^{35,36} and protein disulphide isomerase³⁷, the chaperonins accelerate the rate of folding of other proteins. Unlike *cis-trans* prolyl isomerase and protein disulphide isomerase, whose molecular mechanisms are understood in principle, if not in detail, however, the molecular basis for the rate enhancement observed with the chaperonins is at the moment obscure.

The chaperonin-facilitated reconstitution of Rubisco activity involves two steps—a folding step, followed by the assembly of

two folded but catalytically inactive monomers into a catalytically active dimer. The results presented here are consistent with the notion that the chaperonins facilitate at least some part of the folding step, with assembly of the dimer occurring subsequently and independently of the chaperonins.

The requirement for a large molar excess of chaperonin over the substrate Rubisco-U or Rubisco-A (Fig. 3) is largely due to the unstable nature of the folding intermediate Rubisco-I, generated on diluting Rubisco-U or Rubisco-A into the reaction solution. It is clear from Fig. 4 that the concept of a stable substrate 'patiently' awaiting interaction with sub-stoichiometric quantities of a catalyst does not apply here. Instead, there is an urgent need to stabilize this fickle intermediate by formation of a binary complex with cpn60. A large excess of cpn60 assures the formation of the stable biologically productive binary complex. The need to sequester unstable folding intermediates evidently exists *in vivo* as well as *in vitro*. The formation of a binary complex between the Rubisco large subunit and chloroplast cpn60 was demonstrated long ago^{41,42}. The molar ratio of cpn60 to newly synthesized subunits of Rubisco *in vivo* is also very large^{12,13}.

The molecular details of what happens to Rubisco while it is associated with the cpn60, and the mechanism by which cpn10 and Mg-ATP facilitate the discharge of the binary complex leading to the formation of active Rubisco, are the least understood aspects of chaperonin function. The hydrolysis of ATP seems to be necessary, because non-hydrolysable analogues of ATP do not support reconstitution. Although the stoichiometry of the reaction is unknown, it is unlikely that the chaperonin system is thermodynamically coupled, with the energy released on hydrolysis of ATP being conserved in the folded native state. Instead, we favour the notion that the hydrolysis of ATP serves some regulatory function and that the energy is ultimately dissipated as heat.

The *E. coli* chaperonins clearly did not evolve to facilitate the folding or assembly, or both, of Rubisco, a protein not normally found in *E. coli*. It follows that the interaction between cpn60 and the partly folded Rubisco cannot be specific for Rubisco alone. Instead, the specificity of the interaction must lie in some so far undefined structural element of the partly folded protein. Because the native protein shows no detectable interaction with cpn60, this structural element must be missing altogether from, or inaccessible in, the native protein. The solution to the problems of substrate specificity and reaction stoichiometry, and to the many other questions raised by this study, await further investigation. □

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LETTERS TO NATURE

Lunar basalt breccia identified among Antarctic meteorites

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THE Antarctic meteorite Elephant Moraine (EET) 87521 is a lunar mare basalt breccia. This 30.7-g meteorite was originally identified as a brecciated eucrite¹, but new petrographic evidence from a more representative thin section (Fig. 1) indicates that it is a breccia of mainly very-low-titanium (VLT) basalt, and should be reclassified as a lunar meteorite. The four (or five) Antarctic lunar meteorites collected previously at Allan Hills, Yamato Mountains and MacAlpine Hills (ALHA81005, Y79117/Y793274, Y82192/82193 +/– Y86032(?) and MAC88104/88105) have all sampled only the plagioclase-rich lunar highlands^{2,3} which dominate the surface of the Moon. EET87521 extends the lunar meteorite collections to include the less abundant basaltic provinces (the maria) of the lunar crust.

Meteorite EET87521 contains lithic and mineral clasts in a matrix of fine-grained breccia and glass. The modal mineralogy, determined from 900 random microprobe analyses of thin section, 87521, 8 (area ~90 mm²) is: 10% olivine (Fo₆₂–Fo₃; Fo, forsterite); 47% subcalcic augite and augite with Mg/(Mg + Fe) ratios between 0.65 and 0.2 (Fig. 2); 38% calcic plagioclase (An₆₈–An₉₅; An, anorthite)¹; 4% silica minerals; 1% of ilmenite, chromite, titanium-rich chromite and sulphide. This mineralogy is comparable with many mare basalts⁴. Representative compositions of most minerals are given in Table 1. An estimate of the bulk composition derived from the modal abundance and the

mean compositions of the phases present is: 47% SiO₂, <0.8% TiO₂, 11.6% Al₂O₃, <0.4% Cr₂O₃, 19.5% FeO, 0.3% MnO, 8.8% MgO, 10.1% CaO, 0.3% Na₂O, ~100 p.p.m. K₂O (ref. 5). These data are consistent with derivation from a mare basalt source. On the basis of instrumental neutron activation analysis (INAA) data⁶, EET87521, 6 seems to contain lunar highlands material, represented by higher normative plagioclase and Mg/(Mg + Fe). The meteorite's oxygen isotope signature⁷ is consistent with a lunar origin ($\delta^{18}\text{O} = 5.4$, where $\delta^{18}\text{O} = ((^{18}\text{O}/^{17}\text{O})_{\text{sample}} / (^{18}\text{O}/^{17}\text{O})_{\text{standard}}) - 1$; $\delta^{17}\text{O} = 2.8$; R. N. Clayton and T. K. Mayeda, personal communication).

The lithic clasts in EET87521, 8 range from a 5-mm, fairly coarse-grained gabbroic fragment with fayalite, iron-rich augite, plagioclase and various oxides (Fig. 1) to small fine-grained graphic intergrowths of augite, fayalite and silica in ilmenite. Magnesian components (Mg/(Mg + Fe) of 0.65–0.5) are less common (Fig. 2a) and occur almost exclusively as mineral clasts. All the plagioclase and pyroxene is shocked. The presence of shock glass indicates pressures in excess of 30 GPa but the plagioclase has generally not been maskelynitized (that is, transformed to a glass by shock pressures in excess of 25 GPa). The textures of the lithic clasts indicate that the sample was shocked to less than the 25–30 GPa necessary⁸ for maskelynitization and was invaded by shock glass, perhaps at the time of ejection into an Earth-intercepting trajectory.

The mineralogy of EET87521 differs from the basaltic achondrites with which it was originally identified¹, as it contains much more modal olivine and has abundant subcalcic augite and augite, in addition to pigeonite. The range of mineral compositions is similar to that seen in the polymict achondrites but the Fe/Mn ratios of the mafic minerals (90–100 in olivine and 60–80 in pyroxene) are much higher than those from achondrites (30–53 in olivine and 25–40 in pyroxene⁹). The Fe/Mn

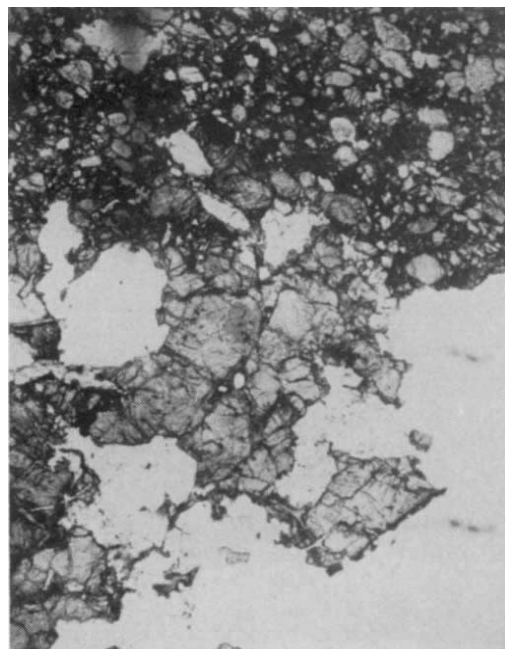


FIG. 1 Augite- and olivine-rich mafic clast and surrounding breccia in meteorite Elephant Moraine 87521, 8, a very-low-titanium basalt breccia. Thin veins of shock glass crosscut both the plagioclase and augite in the lithic clast. Long dimension is ~4 mm, plane-polarized light.

TABLE 1 Representative mineral analyses from EET87521, 8

	Oli1	Oli2	Pyx1	Pyx2	Pyx3	Fel1	Fel2	Fel3	Ilm	Chrm	Ti-sp
SiO ₂	36.6	30.1	52.5	49.6	47.8	45.3	46.3	51.0	0.06	0.02	0.19
TiO ₂	0.03	0.11	0.18	0.39	0.76	bd*	0.01	bd	52.3	1.37	27.9
Al ₂ O ₃	0.05	0.14	1.12	1.13	1.23	36.3	34.4	31.6	0.06	18.7	4.2
Cr ₂ O ₃	0.07	bd	0.59	0.37	0.12	na†	na	na	0.34	45.1	2.57
FeO	33.7	64.0	20.2	26.6	29.5	0.42	0.49	0.77	46.1	27.8	63.1
MnO	0.34	0.63	0.32	0.39	0.35	0.03	bd	bd	0.39	0.21	0.3
MgO	30.7	5.9	19.8	11.1	3.6	0.05	0.06	bd	0.5	5.8	0.23
CaO	0.26	0.38	5.2	7.9	14.7	19.0	17.8	14.0	na	na	na
Na ₂ O	bd	bd	bd	0.06	0.09	0.52	1.02	2.37	na	na	na
K ₂ O	na	na	na	na	na	0.02	0.03	0.33	na	na	na
Sum	101.76	101.28	99.91	97.59	98.08	101.67	100.11	100.05	99.78	99.1	98.46

Oli, olivine; Pyx, pyroxene; Fel, plagioclase; Ilm, ilmenite; Chrm, chromite; Ti-sp, ulvöspinel/magnetite solid solution.

* bd, below detection.

† na, not analysed.

ratios are typical of Apollo and Luna samples and probably of much of the lunar crust. The estimate of the bulk composition resembles the Apollo 17 VLT and Luna 24 VLT basalts¹⁰ but has a low Mg/(Mg+Fe) ratio. The abundant fayalitic olivine in the lithic clasts also shows that this meteorite contains highly fractionated basalts. The incompatible element content (Na and K) seems low in 87521, 8 but rare-earth-element abundances in 87521, 6 (ref. 6) are fairly high and are consistent with a fractionated source.

The number of lunar meteorites from the highlands (four or possibly five) and from the maria (one) matches fairly well with the relative areas of these provinces (83% highlands and 17% maria) on the Moon¹¹. The number of samples is small, but this correspondence suggests that impacts on the Moon that are large enough to propel samples to the Earth have been sufficiently frequent to ensure representative random sampling of the lunar crust. The present lunar meteorite suite is a less biased sampling of the entire lunar crust than the combined Apollo and Luna suites, which together enclose <5% of the surface area of the moon¹¹. The absence of precise locality information for the lunar meteorites restricts the interpretation of the data, but as samples for testing the generality of models

based on the Apollo and Luna suites, they assume enormous importance. Random sampling of the lunar surface by meteorites also implies that two or three of the highlands meteorites are from far-side sites. □

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New double-sheet copper oxide compounds with BiO or TiO bilayers

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ONE of the main families of oxide superconductors comprises compounds with varying numbers of CuO₂ sheets sandwiched between BiO or TiO bilayers^{1–5}. The superconducting transition temperature (*T_c*) in these compounds increases with the number of CuO₂ sheets; for example, *T_c* is 80–108 K for the double-sheet 2212 phase, and 110–125 K for the triple-sheet 2223 phase. Here we describe a new family of double-sheet copper oxide compounds with BiO or TiO bilayers, which can improve our understanding of the relationship between *T_c* and the CuO₂ sheets. The new compounds, Bi₂Sr₂(Ln_{1–x}Ce_x)₂Cu₂O_{10+y} and Ti₂Ba₂(Ln_{1–x}Ce_x)₂Cu₂O_{10+y} (where Ln is Sm, Eu or Gd), have double CuO₂ pyramidal sheets between double BiO or TiO layered units, like the 2212 phase, but with a different arrangement of adjacent CuO₂ sheets. The new bismuth compounds (Bi-2222) have a significantly

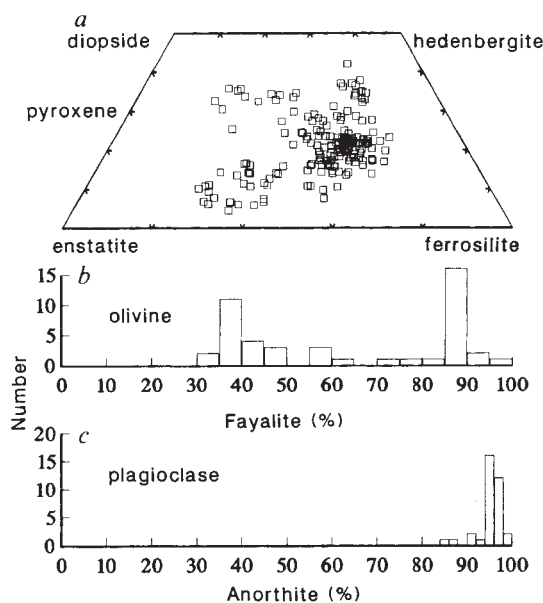


FIG. 2 a, Pyroxene compositions from EET87521. Data obtained from random samples of thin section 87521, 8 and analyses of selected mineral grains. b, Distribution of olivine compositions in 87521, 8. c, Distribution of plagioclase feldspar compositions.

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lower T_c (~ 30 K or less) than the 2212 or 2223 phases, even when the charge-carrier (hole) concentrations are comparable. So far, we have not found superconductivity in the Tl-2222 compounds.

We synthesized the materials by solid-state reaction. For the bismuth-based 2222 compounds, $\text{Bi}_2\text{Sr}_2(\text{Ln}_{1-x}\text{Ce}_x)_2\text{Cu}_2\text{O}_{10+y}$, we ground a mixture of stoichiometric amounts of Bi_2O_3 , SrCO_3 , Ln_2O_3 ($\text{Ln} = \text{Sm}$, Eu or Gd), CeO_2 and CuO and fired it at 900°C for 10 h; we then reground it and refired it at 990°C for 10 h. We pressed the resulting powder into pellets and sintered it at $1,000^\circ\text{C}$ for 10 h. All firing procedures were done in air and followed by slow cooling. According to the X-ray powder diffraction patterns (see Fig. 1), the materials were nearly single phase for cerium concentrations x in the range 0.03–0.25. We found no evidence in the X-ray diffraction patterns for any superconducting T^* phase⁶, Bi-2201, 2212 or 2223 phases. These would complicate our interpretation of the observed superconductivity. We prepared the corresponding thallium-based compounds, $\text{Tl}_2\text{Ba}_2(\text{Eu}_{1-x}\text{Ce}_x)_2\text{Cu}_2\text{O}_{10+y}$, by thoroughly mixing stoichiometric quantities of Tl_2O_3 , Eu_2O_3 , CeO_2 and BaCuO_2 with a 10% excess of Tl_2O_3 . After grinding, we pressed the mixture into a pellet, wrapped it in gold foil, fired it at 890°C for 3 h in a sealed quartz tube containing ~ 1 atm of oxygen, and then slowly cooled it. We readily obtained the desired 2222 phase for compositions near $x = 0.1$, although the resultant material did contain several per cent of the 1212 phase^{4,5}.

We determined the crystallography and microstructure of both 2222 derivatives using X-ray diffraction and transmission electron microscopy (TEM). Tl-2222 is tetragonal (space group $P4/nmm$) with $a = 3.888(2)$ Å and $c = 17.281(7)$ Å; the unit cell is shown in Fig. 2a. Like the 2212 structure (Fig. 2b), the 2222 structure contains two pyramidal CuO_2 sheets between the BiO or TlO bilayers but, instead of a layer of Ca^{2+} ions, there is an intervening fluorite-like $(\text{Ln}_{1-x}\text{Ce}_x)_2\text{O}_2$ layer between the two CuO_2 sheets. As a result, the copper in adjacent CuO_2 sheets are displaced by $(a+b)/2$ in the tetragonal cell. Figure 3 is a high-resolution TEM image along the $[100]$ direction of a $\text{Tl}_2\text{Ba}_2(\text{Eu}_{0.9}\text{Ce}_{0.1})_2\text{Cu}_2\text{O}_{10}$ crystal. The simulated-image inset in Fig. 3, which is based on the unit cell derived from X-ray diffraction data, is in good agreement with the actual image. By contrast to the thallium derivative, Bi-2222 is slightly orthorhombic. The unit-cell axes (Table 1) for this phase (compared to Tl-2222) must be rotated $\sim 45^\circ$ about the c axis and redefined with $a \sim b \sim \sqrt{2}a_p$ (the perovskite unit-cell lattice parameter, a_p is 3.88 Å). Electron diffraction patterns taken on and about the (001) zone axis revealed that there is a strong commensurate superlattice modulation with wavevector $[\frac{2}{9} \ 0 \ \frac{1}{2}]$ in the $\sqrt{2}a_p \times \sqrt{2}a_p$ setting. Given the structural similarity of Bi-2222 and Bi-2212, we speculate that the modulation arises from the insertion of one extra oxygen for every nine bismuth atoms in the

BiO bilayers. Iodometric titration measurements on Bi-2222 are consistent with this hypothesis (see below). The results of the Rietveld analysis on Bi-2222 samples with stoichiometries $\text{Bi}_2\text{Sr}_2(\text{Ln}_{1-x}\text{Ce}_x)_2\text{Cu}_2\text{O}_{10}$ ($x = 0.15$ – 0.25) ($\text{Ln} = \text{Sm}$ or Gd) are listed in Table 1. Unlike 2212 and 2223, neither 2222 derivative contained extensive intergrowths or stacking defects and no twins were observed in cleaved flakes of Bi-2222. We did not perform the neutron diffraction measurement because the compounds contain strongly neutron-absorbing rare-earth elements.

The 2222 structure is closely related to those of well-known bismuth- and thallium-based compounds^{1–5}, but has a different stacking of Cu–O sheets. In the T^* phase⁶ and in the recently discovered Eu–Ba–Ce–Cu–O phase⁷, the fluorite-like layers are sandwiched between K_2NiF_4 -type units and disordered CuO chains, respectively, rather than between BiO and TlO bilayers as in our 2222 phase. Both of these phases have $T_c \sim 25$ – 35 K (refs 6, 7). While preparing this work for publication, we became aware of the discovery⁸ of a (non-superconducting) thallium-based compound with fluorite-like layers, but this structure has TlO monolayers rather than bilayers.

We probed the superconductivity in the Bi-2222 compounds by measuring the Meissner effect. Figure 4 shows the results measured with 10-Oe field cooling for $\text{Bi}_2\text{Sr}_2(\text{Ln}_{0.85}\text{Ce}_{0.15})_2\text{Cu}_2\text{O}_{10+y}$, ($\text{Ln} = \text{Sm}$, Eu and Gd). In all compounds, we observed a substantial Meissner signal with an onset T_c of ~ 25 K and a magnitude $>10\%$ of the ideal value, which ensures the bulk nature of the observed superconductivity. To produce the superconductivity in the Bi-2222 samples, extensive oxygen annealing was necessary. We oxygenated the samples for which the data are plotted in Fig. 4 under 80 atm of oxygen at 550 – 600°C for 10 h. By iodometric titration, we determined the amount of extra oxygen y , which is postulated to reside in the BiO bilayer, to be $y \sim 0.24 \pm 0.02$. For the same oxygenation conditions, the y value was approximately constant for samples with $x = 0.15$ – 0.25 , but decreased for compounds with less cerium. Because the valence of the cerium ion in the fluorite-like layer is very likely⁹ to be $4+$, the effective $[\text{CuO}]^{p+}$ charge or the concentration p of chemically doped holes per

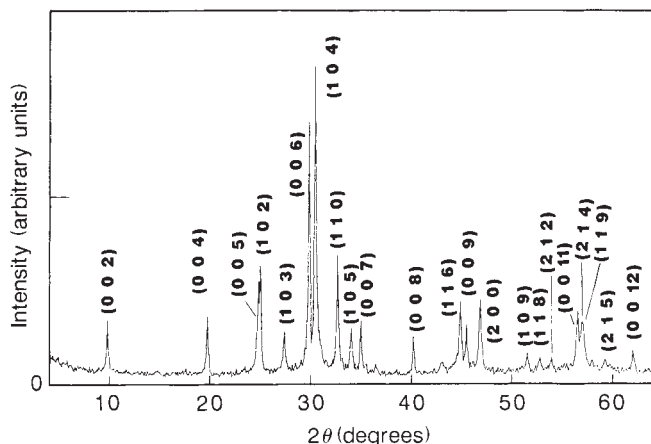


FIG. 1 Powder X-ray diffraction pattern for new Bi-2222 phase.

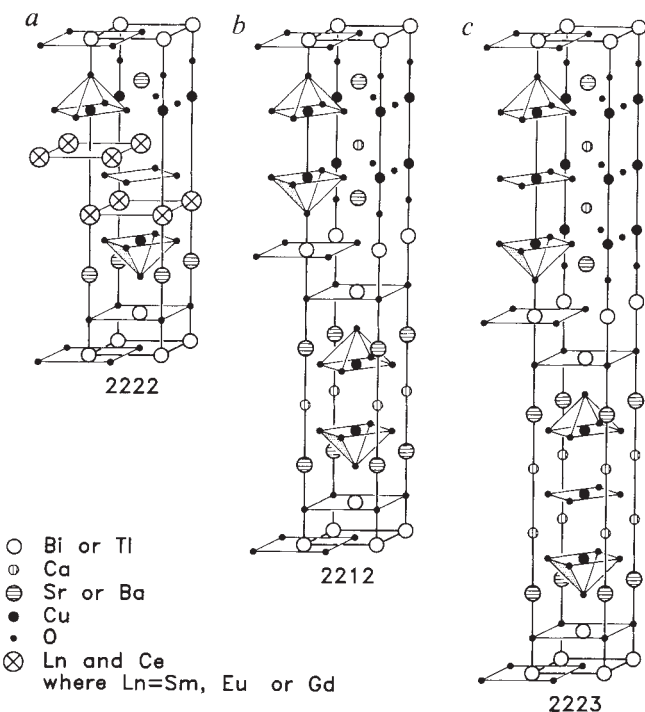


FIG. 2 Nominal unit cells for the a, 2222; b, 2212; and c, 2223 phases.

TABLE 1 Atomic coordinates of the new Bi-2222 compound

	$x=0.25$	$\text{Bi}_2\text{Sr}_2(\text{Sm}_{1-x}\text{Ce}_x)_2\text{Cu}_2\text{O}_{10+y}$ $x=0.20$	$x=0.15$	$\text{Bi}_2\text{Sr}_2(\text{Gd}_{1-x}\text{Ce}_x)_2\text{Cu}_2\text{O}_{10+y}$ $x=0.15$
a	5.4630 (10)	5.4670 (22)	5.4636 (13)	5.4445 (9)
b	5.4730 (10)	5.4708 (22)	5.4723 (13)	5.4573 (9)
c	17.887 (2)	17.919 (2)	17.921 (2)	17.913 (2)
a/b	0.998	0.999	0.998	0.997
Bi	in $(0\frac{1}{4}z)$ $z=0.5854$ (4)	0.5853 (7)	0.5842 (6)	0.5854 (7)
Sr	in $(0\frac{1}{4}z)$	0.2671 (9)	0.266 (1)	0.266 (1)
Cu	in $(0\frac{1}{4}z)$	0.833 (1)	0.823 (2)	0.837 (3)
Sm,Ce	in $(0\frac{1}{4}z)$	0.0687 (6)	0.0693 (8)	0.0679 (8)
O(1)	in $(0\frac{1}{4}z)$	0.399 (5)	0.419 (7)	0.403 (5)
O(2)	in $(0\frac{1}{4}z)$	0.710 (5)	0.715 (6)	0.718 (9)
O(3)	in $(\frac{1}{4}0z)$	0.841 (3)	0.835 (4)	0.841 (3)
O(4)	in $(\frac{1}{4}00)$			
B_{metal} ($\text{\AA}^2$)	2.3 (2)	1.9 (2)	2.0 (2)	2.1 (2)
B_{O} ($\text{\AA}^2$)	4.9 (13)	2.4 (13)	0.2 (12)	2.3 (14)
R_{wp} (%)	11.70	12.58	11.79	11.86
R_{I} (%)	6.58	8.57	6.66	7.80

Space group $Cmma$ with $\sqrt{2}a_p \times \sqrt{2}a_p \times c$ unit cell. The values in parentheses represent the uncertainty in the last digit(s). B_{metal} and B_{O} are the Debye-Waller factors for metal and oxygen, respectively. R_{wp} and R_{I} are the R -factors for the weighted sum and intensities, respectively.

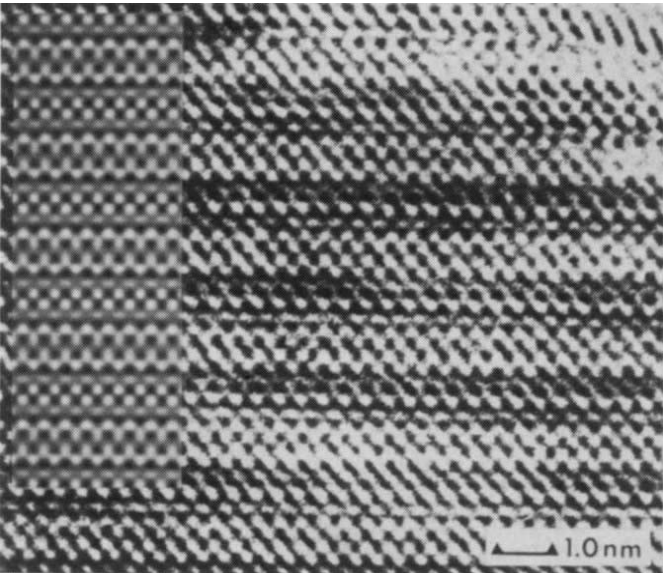


FIG. 3 High-resolution TEM image along the $[100]$ direction of TI-2222 obtained using a Philips 430ST. The inset is a simulated image using 2-nm thickness and ~ 40 -nm delocus.

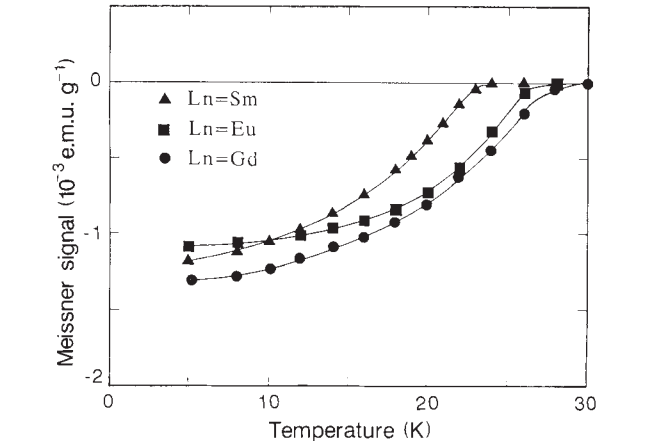


FIG. 4 Meissner signals of Bi-2222 phase compounds, $\text{Bi}_2\text{Sr}_2(\text{Ln}_{0.85}\text{Ce}_{0.15})_2\text{Cu}_2\text{O}_{10.2}$ ($\text{Ln}=\text{Sm}, \text{Eu}$ and Gd). We plot the ratio of magnetization M to applied field H , for $H=10$ Oe. The Curie-like contribution of magnetic lanthanide ions was subtracted.

copper atom is given by the relation, $p = y - x$. The Bi-2222 samples all have positive p values in the range 0–0.1, indicating that the charge carriers are holes and not electrons, by contrast to cerium-doped Ln_2CuO_4 compounds^{9,10}. This is also consistent with preliminary results of thermopower measurements, which show the existence of positive charge carriers. The cerium ions stabilize the fluorite-like Ln_2O_3 layer, as observed in other families of layered copper oxides^{6,9}, and also reduce the total number of holes introduced by the excess oxygen. In fact, highly cerium-doped compounds ($x = 0.25$) showed semiconducting or insulating behaviour in the temperature dependence of their resistivity.

According to iodometric-titration analysis, the above mentioned oxygenation procedure can increase the hole concentration up to $p = 0.10$ in Bi-2222, nevertheless T_c remains around 25 K for $\text{Ln} = \text{Sm}, \text{Eu}$ or Gd . It is more difficult to characterize the stoichiometry or control the oxygen content of TI-2222 than it is for Bi-2222, but according to our preliminary data the samples show metallic transport behaviour.

The low T_c observed in Bi-2222 is in sharp contrast to the higher T_c s observed in the 2212 compounds (80–108 K) and the 2223 compounds (110–125 K) with comparable values of p (≈ 0.1 –0.2). We note that the 2222 and 2223 compounds have a repeat unit of nearly the same size between the BiO or TlO bilayers: the fluorite-like layer in the 2222 phase contains one O_2 sheet sandwiched by two lanthanide layers, which corresponds well to the CuO_2 sheet between the calcium layers in the 2223 phase (Fig. 2a, c). According to the empirical law for charge-carrier types^{9,10}, an isolated CuO_2 sheet with no apical oxygen cannot sustain holes as charge carriers but can be doped with electrons, whereas the reverse is true for the pyramidal CuO_2 sheet. Therefore, the hole-type carrier motion is probably primarily restricted to the pyramidal CuO_2 sheets in 2223. The distance $d = 5.9 \text{ \AA}$ between the hole-conducting pyramidal CuO_2 sheets in 2222 ($T_c = 25 \text{ K}$) is comparable to that ($d = 6.4 \text{ \AA}$) in 2223 ($T_c = 110$ –125 K). Why then is T_c so low in the 2222 compound? One of the most significant differences between the 2222 compound and the 2212 or 2223 compounds may be the relative arrangement of the adjacent pyramidal sheets (Fig. 2). Another factor may be the larger in-plane Cu–O bond lengths¹¹ (induced by the presence of the fluorite-like layers) in the 2222 compounds, which might decrease T_c . Instead of depending simply on the number of Cu–O sheets between the BiO and TlO bilayers, it seems that T_c depends strongly on a combination of factors, including the stacking pattern of these sheets, the Cu–O bond lengths and the carrier concentration. □

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Wave damping by rain

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IT is common knowledge among seafarers that rain “knocks down the sea”^{1,2} or that surface-wave breaking is suppressed by heavy rain. Although no quantitative data exist, several mechanisms have been proposed that might account for the attenuation of waves by rain. The most likely seems to be that raindrops produce turbulence in the water which then helps to dissipate the waves. Here we describe laboratory experiments which show that, in the absence of wind, rain preferentially damps short-wavelength gravity waves. The observed damping rates are, however, lower than earlier predictions^{3,4}. The suppression of short waves has an effect on the sea surface similar to that of an oil film, leading to a reduction in the breaking of long waves. The phenomenon is important because it helps to provide insight into other aspects of wave breaking or wave suppression, and because of its bearing on the detection and estimation of rainfall at sea from ambient noise levels⁵, and of the measurement of winds using radar, as scatterometer estimates of wind speed are known to be biased when it rains⁶. Wind speed is determined from an empirical relation with the observed strength of the backscattered radar signal. Although the signal may be influenced by atmospheric effects in rain, the very-small-scale roughness of the surface caused by rain drops or, as we shall show, the reduction of the 10-cm-scale waves, may also affect the signal and hence the wind-speed estimate.

Observations⁷ and analytical studies⁸ have shown that, in windy conditions, rain may enhance the net stress of the wind on the sea surface and, at first sight, suppression of wave breaking is therefore unexpected. The phenomenon is, however, far from simple. Rain modifies the structure of the atmospheric boundary layer⁹. The differential velocity of raindrops striking the moving sea surface induces both normal and tangential stresses on the water³. The net stress contributes both to waves and mean currents. The impact of raindrops produces capillary gravity ripples¹⁰, sub-surface vortex rings² and vertical mixing^{3,4},

bubbles^{11,12} and the generation of secondary drops—for example, when large bubbles burst on returning to the surface. A thin fresh-water mixing layer may build up at the sea surface¹³, with density gradients restricting diffusion¹⁴.

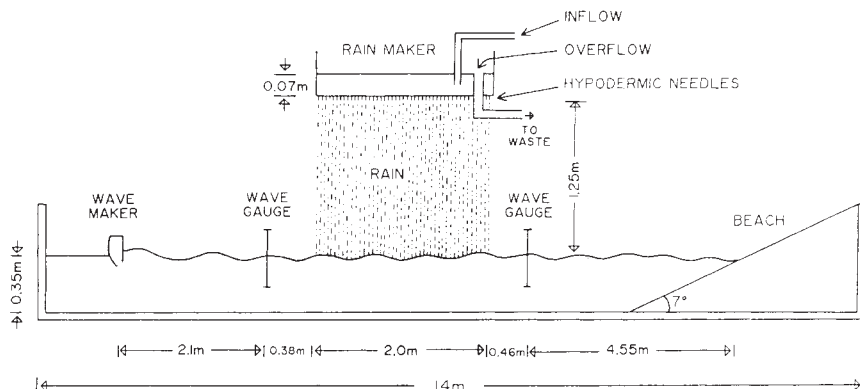
The full problem is very complex and we therefore conducted laboratory experiments in which only the most essential elements are retained—the rain (all of the same drop size) and the waves on fresh water—neglecting, for the present, the added complexity of wind and salinity. We used a wave tank that is 14 m long, 0.6 m wide and 0.6 m deep (Fig. 1). We produced drops of radius $R = 1.6$ mm with an array of 3,200 hyperdermic needles mounted 25 mm apart, in a triangular pattern and set in the base of waterfilled trays mounted 1.25 m above the water surface. The ‘artificial rain’ covered a 2-m-long section spanning the wave tank. Drops reached the water surface with a fall speed W of 4.5 m s^{-1} , about half their terminal fall speed¹⁵, but with an intensity I of 600 mm hr^{-1} , greater than usual under natural conditions. The net flux of kinetic energy per unit area of the water from the artificial rain, $\frac{1}{2}\rho W^2 I$, where ρ is the density of water in raindrops, was comparable to that of natural rain. We produced waves of frequency $\sigma = 1.8$ – 4.2 Hz using a wedge at one end of the tank. These waves passed through a capacitance wire gauge, through the ‘rain’ section and then through a second gauge before finally being absorbed on a ‘beach’. In our first, ‘no-rain’, experiments we used the gauge outputs averaged over 40 waves to determine a damping coefficient δ (a function of σ , or of wavenumber k) from $a_2 = a_1 \exp(-\delta d)$, where a_1 , a_2 are the wave amplitudes at the first and second gauges and d is their separation. Damping is the result of viscous dissipation at the sides and bottom of the tank¹⁶, and in the interior and at the surface of the fluid¹⁷. We found the second source of damping to be dominant, with a value larger than that of molecular viscosity at a clean water surface

$$\delta = 4\sigma^5 \nu / g^5 \quad (1)$$

(where g is the acceleration due to gravity), but well below that of a surface at which tangential motion is entirely suppressed (Fig. 2). (As the water surface was not ‘pre-cleaned’ we expected some slight contamination.) At frequencies $\leq 16 \text{ rad s}^{-1}$ (2.5 Hz), measurements were affected by wave reflection from the beach and the presence of cross-waves. Measurements of short waves (frequencies $> 25 \text{ rad s}^{-1}$ or 4 Hz) were affected by errors due to capillary effects at the wave gauges.

Our experiments with rain showed enhanced damping. To derive the attenuation rate Δ for surface waves in the rain (Fig. 3a), we used the attenuation rate δ to account for wave damping in the area between the gauges but outside the 2-m rain zone, and also corrected for side and bottom damping in the rain zone. We have estimated the damping owing to (1) the pressure of the rain on the advancing waves, (2) the tangential stress on the moving water surface that is due to the relative speeds of rain drops entering the water, and (3) the momentum loss of the waves through the interaction with capillary gravity waves produced by raindrops is not appreciable. This net contribution,

FIG. 1 The experimental apparatus. Artificial rain is produced from an array of hypodermic needles set in the bottom of a tray and falls onto waves produced by a wavemaker. The wave gauges are 0.315-mm-diameter capacitance wire probes located on the centre line of the tank.



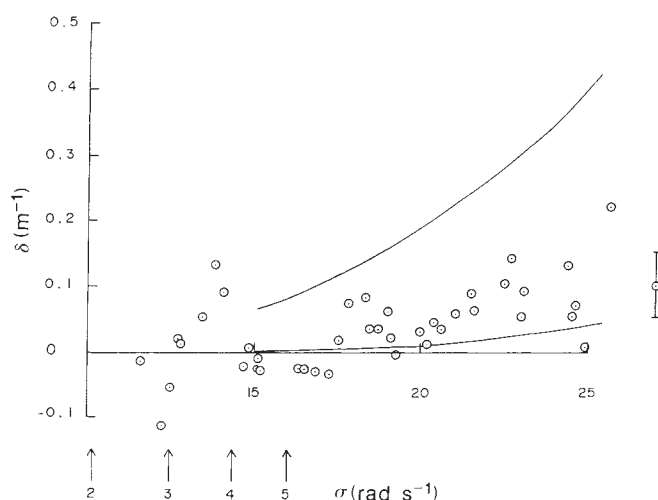


FIG. 2 The damping rate δ as a function of frequency σ of waves in the absence of rain. We have corrected the data for the effects of side walls and tank bottom. The vertical bar indicates the possible uncertainty associated with each estimate. The arrows marked 2-5 indicate the frequencies at which cross-waves may occur; the number is the mode number. The lower curve is the predicted curve with a free upper surface and $\nu = 1.1 \times 10^{-6} \text{ m}^2 \text{ s}^{-1}$. The upper curve is that predicted for a contaminated water surface with no tangential motion possible.

assuming the molecular viscosity and no turbulent motion, is $\leq 10\%$ of the observed damping. By analogy with equation (1) the damping by vertical mixing may be represented

$$\Delta = 4\sigma^5 \nu_e / g^3 \quad (2)$$

where ν_e is an effective viscosity. Figure 3b shows values of ν_e derived from equation (2), which have a mean value of $2.84 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}$ for σ in the range 16.0–25.2 rad s^{-1} . The curve in Fig. 3a shows Δ as a function of σ for this mean value. We found no significant dependence of ν_e on wave steepness ($0.05 < a_1 k < 0.3$).

Yakimov¹⁰ has suggested $\nu_e = bLI[1 - \exp(-2\sigma^2 L/g)]$, where b is estimated to be ~ 15 and represents the mass carried down in the water by each raindrop divided by the mass of the drop, and L is the depth to which mixing extends, assumed¹⁰ to be $\sim 5 \text{ cm}$. Agreement with the observed ν_e requires $L = 1.2 \text{ cm}$, which is less than the observed mixing-layer thickness; the formula seems to overestimate ν_e . ($L = \nu_e / I$ corresponds to the boundary-layer thickness predicted for flow at rate I through a porous wall into a fluid of viscosity ν_e . Using our experimental parameters, $L = 17 \text{ cm}$, which is not inconsistent with depths observed when artificial rain is dyed.) Manton's³ proposal, $\nu_e = RI^{1/6}W^{5/6}$, gives ν_e in terms of known parameters but it overestimates ν_e ($1.31 \times 10^{-3} \text{ m}^2 \text{ s}^{-1}$).

We can judge the possible importance of rain damping in modifying the wave field by comparing the observed Δ with the growth rate of waves that is due to wind¹⁸, $\beta = 0.04k(u_*/c)^2$, where u_* is the friction velocity, which is ~ 0.04 times the wind speed 10 m above the water surface, W_{10} , and $c = \sigma/k$ is the phase speed. Assuming the gravity-wave dispersion relation, $\sigma^2 = gk$, β and Δ (given by equation (2)) are equal when $\sigma\nu_e = 1.6 \times 10^{-5} W_{10}^2$. In a wind of 6 m s^{-1} , damping at the rate observed in the experiments would exceed the growth induced by the wind for waves of frequency $> 20 \text{ rad s}^{-1}$ (wavelength $\leq 0.16 \text{ m}$).

A further contribution to short-wave dissipation in windy conditions is due to the enhancement of wind stress by rain^{7,8} and a consequently increased surface wind drift current. The rain-induced stress $\tau_r \approx 0.85\rho IW_{10}$ (ref. 8), together with the wind stress τ lead to a surface drift q proportional to $[(\tau + \tau_r)/\rho]^{1/2}$, which increases with rain intensity. This then leads to a reduction in the amplitude, $a = (c^2/2g)(1 - q/c)^2$, at which short gravity waves break¹⁹. This small-scale breaking represents

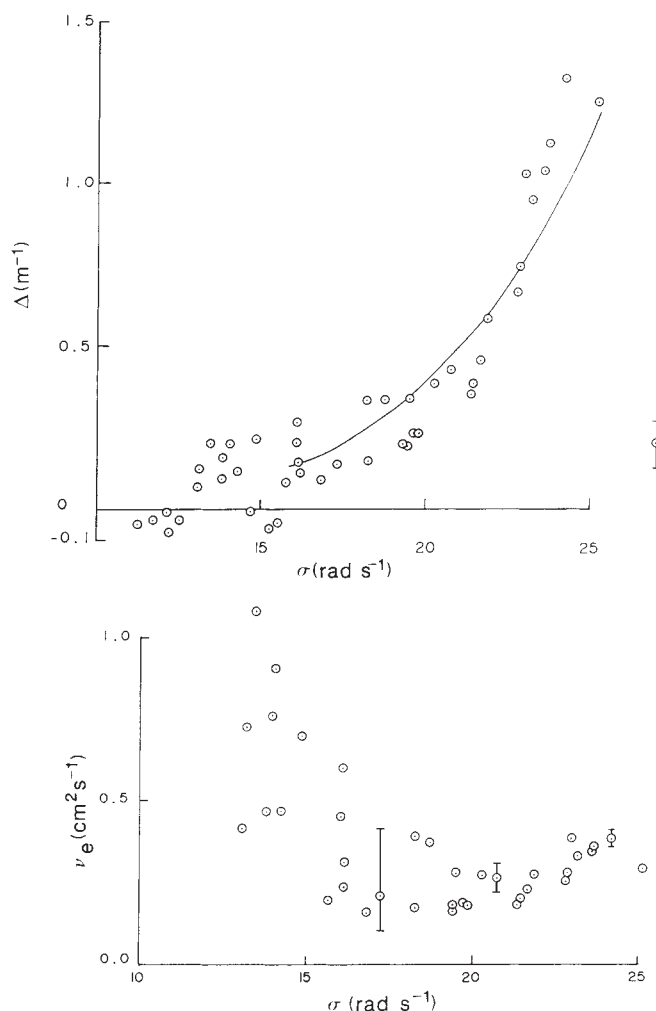


FIG. 3 a, The damping rate Δ as a function of frequency σ of waves in rain, with side-wall and tank-bottom effects removed. The vertical bar indicates the possible uncertainty associated with each estimate, and the curve is that given by equation (2) with the average value of ν_e . b, The effective viscosity ν_e as a function of wave frequency σ , using only values of $\Delta > 0$ in a. The vertical bars indicate the possible uncertainty associated with each estimate. Points for $\sigma < 16 \text{ rad s}^{-1}$ are seriously affected by cross-waves and edge-waves at the beach and should be discounted. There seems to be no significant trend in ν_e for σ in the range 16–25.2 rad s^{-1} .

an increased rate of energy loss from the short waves, and to waves of smaller mean amplitude.

Short waves with $k > g/(25u_*^2)$, which include those severely damped by rain, constitute a major component of the roughness of the surface, which is important in the transfer of momentum from the wind to the larger waves²⁰. Although raindrops, and splashes, roughen the sea surface at the scales of capillary gravity waves, the attenuation of the short gravity waves caused either by the enhanced turbulence observed in experiments or by their dissipation through small-scale breaking as indicated above, will reduce the surface roughness and hence the momentum transfer to the longer waves, and so diminish the frequency of the more violent form of breaking associated with the longer waves, which are a hazard to mariners. By reducing the short waves, rain calms the sea in a way similar to oil²¹. In the absence of wind, as in the experiments, heavy rain tends rapidly to attenuate short waves typical of lakes or fjords, and contributes to the glassy appearance of their surfaces commonly observed once the rain has stopped. Further experiments to determine the functional relation of I and W are necessary, as is a comprehensive study of rain and wave interactions in windy conditions⁶. □

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Nitrification rates and ^{15}N abundances of N_2O and NO_3^- in the western North Pacific

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NITROUS oxide plays an important part in atmospheric chemistry; it is a 'greenhouse' gas and has the potential to destroy ozone. Concentrations of N_2O in surface waters of the oceans are generally close to equilibrium with the atmosphere, but sub-surface sea water is supersaturated with N_2O ^{1–5}. The oceans are now regarded as a source⁴ for atmospheric N_2O by production through the oxidation of ammonium ('nitrification')^{1,3}, except in extremely oxygen-deficient waters⁶. Nevertheless, the possibility that N_2O might be produced through the reduction of nitrate

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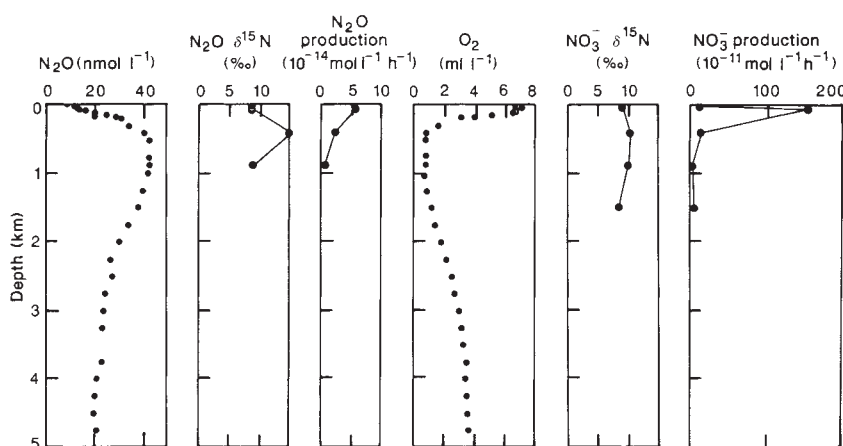
('denitrification') cannot be ruled out^{5,7}. The distribution of ^{15}N in atmospheric and oceanic N_2O can be used to investigate the behaviour of N_2O in the oceans^{8,9}. Here we report simultaneous measurements of the nitrification rates and nitrogen isotope data for N_2O and NO_3^- produced in the western North Pacific Ocean. The amount of N_2O produced by nitrification is much lower than that expected from the production of NO_3^- . The N_2O in the oxygen-deficient layer is more enriched in ^{15}N than NO_3^- . These results imply that the contribution of nitrification to the production of N_2O is lower than previously thought and that denitrification is primarily responsible for the production of N_2O .

During a cruise (August–September 1983) of the RV *Hakuho Maru* in the western North Pacific Ocean we collected seawater samples at depths ranging from the surface to 4,800 m at Station C (45° 09' N, 160° 18' E) and from the surface to 4,000 m at Station E (28° 06' N, 152° 56' E). Figures 1 and 2 show the vertical profiles of the N_2O concentration, $\delta^{15}\text{N}$ values and the potential production rate of N_2O and NO_3^- by nitrification and the dissolved oxygen at Stations C and E, respectively. The dissolved N_2O concentrations in surface water (9.0 nM at Station C and 5.6 nM at Station E) agree well with the equilibrium values estimated using solubility data of N_2O (ref. 13). The maximum concentrations of N_2O occur at a depth of 700 m at Station C and at 1,300 m at Station E, where the dissolved oxygen is a minimum. There is a strong correlation between the N_2O and NO_3^- concentrations and a negative correlation between the N_2O and O_2 concentrations at both stations, as shown in Fig. 3. These correlations are similar to those reported for other areas of the oceans^{1–5,7} except for the deep-water column at Station E where a water mass enriched in NO_3^- and depleted in N_2O (ref. 4) is mixed in. The lines in Fig. 3 'turn over' because of the depth dependence— N_2O is generally depleted in bottom waters.

The $\delta^{15}\text{N}$ value of the dissolved N_2O in surface water is

FIG. 1 The vertical profiles at Station C of (from left to right) N_2O , $\delta^{15}\text{N}$ of N_2O , the potential production rate of N_2O by nitrification, dissolved O_2 , $\delta^{15}\text{N}$ of NO_3^- and the potential production rate of NO_3^- by nitrification.

METHODS. We determined the N_2O concentration of the water samples, which had been stored in 100-ml air-tight glass containers, using the method given in ref. 10. We determined using a method we have described elsewhere⁹, the ^{15}N content ($\delta^{15}\text{N} = ((^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{standard}}) - 1$, the standard is N_2 purified from the atmosphere) of N_2O in 100-l samples collected at several depths at the two stations; to determine the $\delta^{15}\text{N}$ of NO_3^- we used the method in ref. 11 (5-l samples). We estimated (on board) the production rates of N_2O and NO_3^- by nitrification using ^{15}N -tagged NH_4^+ . We added 5 μmol of NH_4^+ (99.8 atom% ^{15}N) to a 5-l water sample contained in an air-tight vessel. After incubating the sample at approximately the *in situ* temperature for 48 h (in the dark), we added 5 ml of 0.1M HgCl_2 to stop the bacterial reactions. We used the same method to remove the N_2O as we used to remove N_2 having a natural abundance of ^{15}N . We determined the $\delta^{15}\text{N}$ of the 50–200 nmol N_2O so collected by adding a known amount ($\sim 1 \mu\text{mol}$) of N_2O having $\delta^{15}\text{N} = -2.9\%$. We measured the $\delta^{15}\text{N}$ of NO_3^- using the same method as we used for NO_3^- having a natural ^{15}N abundance. At the start of the



incubation the NH_4^+ is $\sim 1 \mu\text{M}$, about ten times larger than the saturation concentration for nitrification¹². The rates that we calculate correspond to the potential production rates of N_2O and NO_3^- . The precision of the measurements are $\pm 2\%$, $\pm 0.2\%$ and $\pm 3\%$ (1 σ) for N_2O , $\delta^{15}\text{N}$ and production rates, respectively.

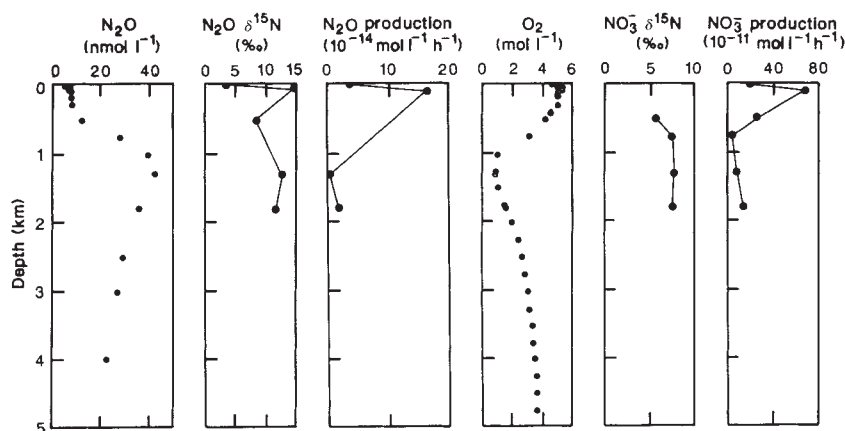


FIG. 2 The vertical profiles at Station E of (from left to right) N_2O , $\delta^{15}\text{N}$ of N_2O , the potential production rate of N_2O by nitrification, dissolved O_2 , $\delta^{15}\text{N}$ of NO_3^- and the potential production rate of NO_3^- by nitrification.

identical with that in equilibrium with the atmosphere (the average nitrogen isotope ratio of N_2O in maritime air collected on this cruise is $6.8 \pm 0.7\text{‰}$) at Station C. We measured the lowest $\delta^{15}\text{N}$ value, 3.4‰, at a depth of 10 m at Station E where we observed moderate nitrification activity. The $\delta^{15}\text{N}$ value of the dissolved N_2O and the N_2O concentration have similar vertical profiles. Although N_2O produced by nitrification is very depleted in ^{15}N (ref. 14), we find that the observed N_2O in shallow waters is enriched by ^{15}N , with the exception of the sample from 10-m depth at Station E.

Our method for measuring the N_2O production by nitrification is successful, unlike conventional methods, which cannot measure the increase in N_2O concentration even for the sample of highest activity ($\leq 10^{-11} \text{ mol l}^{-1}$ increase after a 48-h incubation). The potential N_2O production rate by nitrification is highest in the upper aphotic layers and is significantly lower below 500 m. The maximum occurs at the NO_3^- -maximum layer at each station. We do not believe that our method underestimates the potential rates for the samples at depths > 500 m,

because similar or slightly lower nitrogen metabolic activities were reported for the samples incubated at the high pressure characteristic of over 5,000-m depth than for those incubated at sea-level pressure¹⁵. The effects of the pressure difference on the nitrification rate, however, should be tested at some point.

The vertical profiles of the potential rate indicate that nitrification is the major pathway for the production of N_2O in shallow aphotic layers. The potential N_2O production rate by nitrification ranges from 0.5 to $16 \times 10^{-14} \text{ mol l}^{-1} \text{ h}^{-1}$. These values are almost an order of magnitude lower than the values expected from the analogous production rate of NO_3^- —but the NO_3^- production rates are in the range of previous studies^{12,16–20}. If N_2O were produced only by nitrification, the production rate ratio of N_2O and NO_3^- should be 1.1×10^{-3} and 0.8×10^{-3} at Stations C and E, respectively (calculated from the data shown in Fig. 3). From N_2O and NO_3^- yields during the simultaneous incubation we calculate this ratio to be $0.04\text{--}0.19 \times 10^{-3}$ and $0.08\text{--}0.27 \times 10^{-3}$ at Stations C and E, respectively.

Nitrification seems to account for only one-tenth to one-fifth of the N_2O produced. The correlation between $\Delta\text{N}_2\text{O}$ (the difference between the measured concentration and *in situ* saturation concentration of N_2O) and apparent oxygen utilization AOU (the difference between the *in situ* saturation concentration and the measured concentration of dissolved oxygen) cannot be explained solely by nitrification, because the production ratio of N_2O and NO_3^- changes greatly with dissolved oxygen level^{21,22}. It may be explained, however, by denitrification of NO_3^- . Denitrifiers reduce NO_3^- only under anaerobic conditions. Such conditions can be found in the interior of the particulate organic matters such as detritus, fecal pellets and marine snows²³. The NO_3^- concentration increases with decreasing dissolved oxygen, and a low dissolved-oxygen concentration favours the occurrence of anaerobic microsites in which NO_3^- can be reduced to produce N_2O . Consequently, excess N_2O

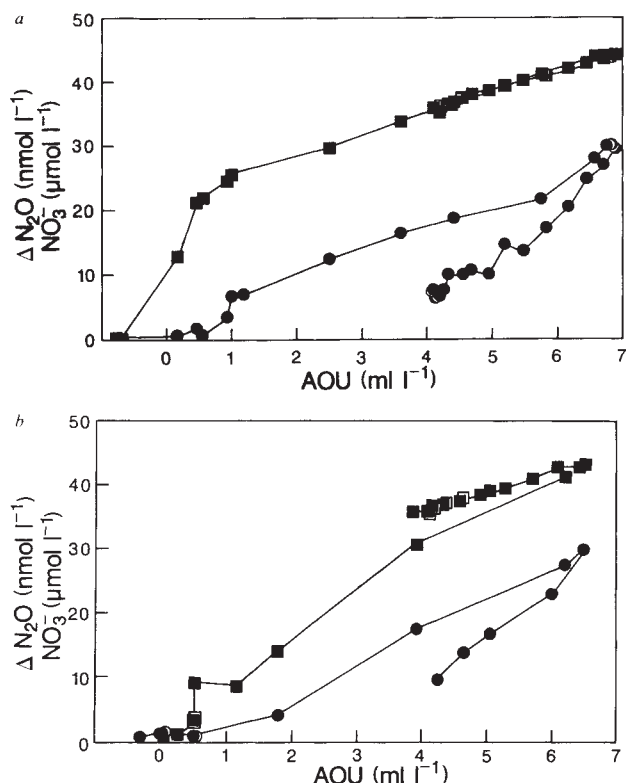


FIG. 3 Relationship between $\Delta\text{N}_2\text{O}$ (circles) and AOU and between NO_3^- (squares) concentration and AOU *a*, at Station C and *b*, at Station E. We used the method in ref. 25 to determine the NO_3^- concentration.

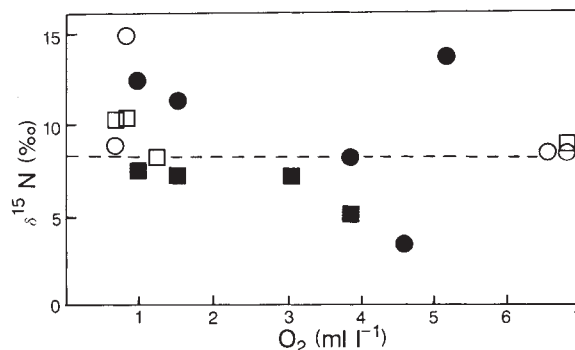


FIG. 4 Relationship between nitrogen isotope ratios of N_2O and NO_3^- and dissolved oxygen concentration. Open circles and squares indicate $\delta^{15}\text{N}$ values of N_2O and NO_3^- at Station C and solid ones, those at Station E, respectively. The dotted line refers to $\delta^{15}\text{N}$ of dissolved N_2O in equilibrium with atmospheric N_2O .

concentration would be proportional to the apparent oxygen utilization.

The nitrous oxide at the concentration maxima of both stations is found to be more enriched in ^{15}N than that in equilibrium with atmospheric N_2O and NO_3^- . The enrichment in ^{15}N of N_2O that we found has been observed in the oxygen-minimum layer of the eastern tropical Pacific Ocean, where denitrification prevails⁹. The $\delta^{15}\text{N}$ values of N_2O correlate with those of NO_3^- (Fig. 4). This indicates that most of the N_2O is involved in the NO_3^- reduction system. Nitrous oxide is consumed as well as produced

in the NO_3^- reduction system. As a result, the $\delta^{15}\text{N}$ of N_2O is determined by the fractionation factors during both production and consumption. We have observed a kinetic nitrogen isotope fractionation factor during the reduction of N_2O by denitrifying bacteria of up to 1.028 in terms of the rate-constant ratio of the isotope species, $k(^{14}\text{N})/k(^{15}\text{N})$ (ref. 24). If the ^{15}N enrichment of substrates is greater during consumption than during production, the enrichment in ^{15}N of N_2O relative to NO_3^- should result. The concentration and the $\delta^{15}\text{N}$ value of N_2O are consequently determined by those of NO_3^- . □

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Evidence for a change in the periodicity of tropical climate cycles at 2.4 Myr from whole-core magnetic susceptibility measurements

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THE Earth's orbit about the Sun is influenced by gravitational interactions with the Moon and other planets. The resulting orbital perturbations give rise to cyclical variations in orbital eccentricity, obliquity and precession with periods of 100, 41, 19 and 23 kyr, respectively¹. These variations are climatically important because they affect the seasonal and latitudinal distribution of solar radiation. Magnetic-susceptibility measurements in deep-sea sediment cores can be used as a sensitive indicator of temporal variations in the concentration of terrigenous material supplied to the sea bed. Here we use this approach to study the evolution of cycles of terrigenous sedimentation at Ocean Drilling Program sites in the Arabian Sea and the eastern tropical Atlantic. We show, from spectral analyses of the two susceptibility time series spanning the past 3.2–3.5 Myr, that the records are driven strongly by orbital

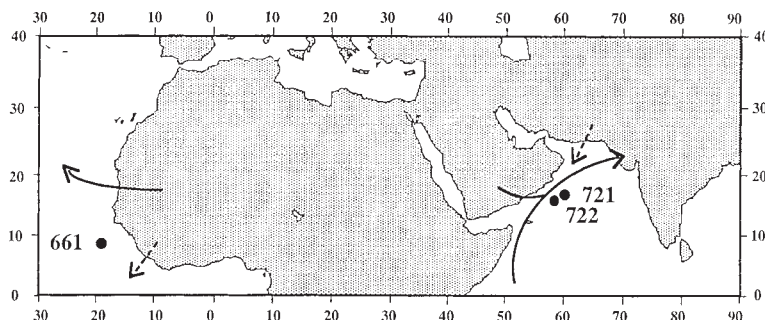
forcing. Before 2.4 Myr before present (BP), both records are strongly influenced by the 23- and 19-kyr periodicities; but after 2.4 Myr, they are both significantly influenced by the 41-kyr periodicity. This shift coincides with the beginning of major Northern Hemisphere glaciation, which indicates, for the Indian Ocean site, that the supply of terrigenous sediment (carried by monsoonal winds) has responded to the rapid increase in ice cover at this time. We cannot, however, exclude possible effects of changes in bottom-water circulation on the susceptibility record of the Atlantic site.

Statistical analyses of the foraminiferal $\delta^{18}\text{O}$ record from deep-sea cores (largely a measure of glacial ice volume²) demonstrate that the growth and decay of ice sheets have been strongly modulated by orbital insolation variations³. Similarly, other major components of climate^{4–8} vary predominantly at orbital periodicities. However, measurement of conventional palaeoclimatic indicators (for example, oxygen and carbon isotopes, carbonate, opal and terrigenous fraction content) in deep-sea sediments is time consuming; consequently, palaeoclimate indicators that can be measured more easily are needed.

Magnetic susceptibility is the ratio of induced magnetization to an applied weak magnetic field and is proportional to magnetic mineral concentration, which is typically a trace component of the terrigenous fraction^{9–11}. Susceptibility measurements are extremely rapid, and whole cores can be measured continuously using a pass-through loop sensor. Provided that susceptibility is correlated to terrigenous-fraction variations, these measurements can offer a highly efficient means of acquiring high-resolution palaeoclimate data.

The major aims of Ocean Drilling Program Legs 108 (eastern tropical Atlantic) and 117 (Arabian Sea) were to study the late Neogene evolution of northwest African climates and the Asian

FIG. 1 Locations of ODP Leg 108 Site 661 and ODP Leg 117 Site 721. Solid arrows depict the modern summer monsoonal dust trajectories, dashed arrows depict the winter trajectories^{13,17}.



monsoon. In the case of the Asian monsoon, summer heating result in the development of an intense low-pressure cell over the Tibetan Plateau; consequently, in the northwest Arabian Sea, strong moisture-laden southwesterly winds transport abundant aeolian detritus¹² to the Arabian Sea region and bring monsoon rains to southern Asia¹³. The northwest African monsoon is driven by the same mechanism: summer heating of North Africa drives the cyclonic inflow of moisture-laden air into the Sahel and south Sahara.

Both general circulation model (GCM) experiments¹⁴ and analyses of aeolian and biogenic components in deep-sea cores^{5,6,14} have demonstrated that seasonal insolation variations resulting from orbital precession have modulated the intensity of the Asian and African monsoons. Precession affects low-latitude summer insolation at periodicities of 23 and 19 kyr (ref. 1); maximum summer insolation is achieved when the summer solstice coincides with perihelion, and palaeoclimate data have demonstrated that the African and Asian monsoon systems were intensified at these times^{5,6}.

Site 721 (Fig. 1) is located in the Arabian Sea in water of depth 2,028 m near the crest of the Owen Ridge, which rises 2,000 m above the surrounding bathymetry. Satellite imagery¹⁵ and sediment-core studies^{15,16} demonstrate that terrigenous sedimentation in the Arabian Sea is dominantly aeolian. Site 661 (Fig. 1) is located in 4,013-m water depth on the eastern slope of the Kane Gap in the eastern tropical Atlantic. Today the area receives a substantial supply of aeolian dust from the African winter dust plume¹⁷. The fact that it is not situated on a bathymetric high suggests that carbonate dissolution and advective sediment supply may have been significant sedimentary processes.

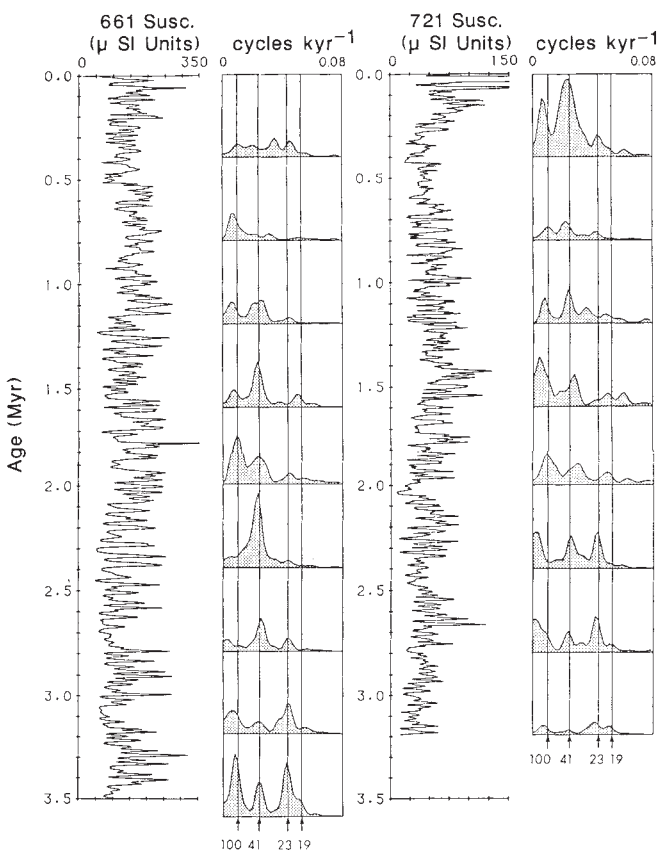


FIG. 2 Susceptibility (Susc.) time series for Site 661 (eastern equatorial Atlantic) and Site 721 (Arabian Sea). The time series were divided into 0.4-Myr intervals and spectral analysis (Sande-Tukey radix-two fast fourier transform with modified Daniell smoothing²¹) was performed on each of these intervals. The vertical lines represent (from left to right) the 100-kyr, 41-kyr, 23-kyr and 19-kyr orbital periodicities. Note the increase in power at the 41-kyr periodicity at both sites after ~2.4 Myr.

At both sites whole-core volume susceptibility was measured at 3–5 cm intervals during the occupancy of the site. Between-hole correlations¹⁸ using the susceptibility data were used to construct complete composite sequences. Age models were constructed using bio- and magnetostratigraphic data^{19,20}. At Site 721 oxygen isotope data (W. L. Prell, unpublished data) were used to construct age–depth relations for the interval 0–1 Myr. The age models included as many data as possible within a series of straight-line segments. The record for Site 661 extends to 3.5 Myr and that for Site 721 extends to 3.2 Myr. Each time series was divided into 0.4-Myr intervals and spectral analysis²¹ was performed on each interval. The resulting periodograms clearly demonstrated that variance was concentrated at orbital periodicities; minor adjustments were then made to some data (within their uncertainty limits) to improve the alignment of the peaks with the orbital periodicities.

Figure 2 shows the susceptibility time series and periodograms. The periodograms indicate a shift in spectral character at ~2.4 Myr. Before 2.4 Myr (2.4–3.5 Myr at Site 661; 2.4–3.2 Myr at Site 721), the data vary predominantly at the 23- and 19-kyr precessional periodicities. In each periodogram, the variance associated with this precession component significantly exceeds that associated with obliquity. After 2.4 Myr there is an increase in variance at the 41-kyr periodicity at both sites, and a corresponding reduction in variance at the 23- and 19-kyr periodicities. At Site 721, increased variance at the 41-kyr periodicity persists over the entire 0–2.4-Myr interval. At Site 661, the periodograms for the interval 0.0–0.8 Myr do not show variance at orbital frequencies; this may reflect an inadequate age model for this interval.

To examine the coherency between the susceptibility records and the orbitally driven insolation variations, the time series were filtered using a bandpass filter centred at 22 kyr, corresponding to orbital precession. The extracted component was then correlated ('tuned') to the calculated precession curve¹ using an inverse-correlation method^{3,22}. Although coherency between the filtered susceptibility data and precession was not evident for Site 661, the filtered susceptibility data at Site 721

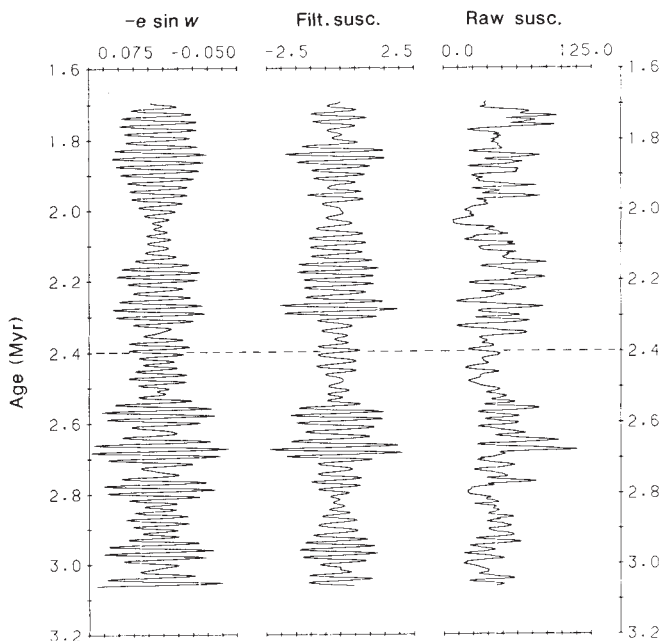


FIG. 3 Calculated orbital precession index ($-e \sin w$), the 22-kyr filter of the susceptibility record and the raw susceptibility record for the 3.2–1.6-Myr interval at Site 721. The filtered data exhibited the modulated character of the precession record, and were therefore correlated ('tuned')³ to the precession curve using an inverse-correlation method²². The 'tuned' age–depth data are within the uncertainties of the original age–depth model²³. The coherency over the entire interval is 0.86; coherency from 3.2–2.4 Myr is 0.89.

exhibits the modulated character of the precessional curve (Fig. 3). Coherency over the 3.2–1.6-Myr interval is 0.84 and is even higher (0.86) from 3.2–2.4 Myr (ref. 23). Although the tuning procedure maximizes the correlation between the two signals, the coherency of the 400- and 100-kyr modulation envelopes is a real component of the susceptibility data. These data document the oldest and longest sequence demonstrating both power and coherency at the precessional band. We consider that these results demonstrate the strong orbital control of the susceptibility

records at both sites, together with a shift from strong precessional forcing before, and strong obliquity forcing after, ~2.4 Myr. This raises the questions of what aspects of the relevant climate systems do the susceptibility records reflect; and what is the significance of the periodicity shift at 2.4 Myr?

Terrigenous extraction procedures²⁴ were conducted on 94 samples from Site 721 (ref. 23) and 64 samples from Site 661. These data (Fig. 4a, b) show that susceptibility is an accurate proxy indicator of terrigenous content at both sites. Using the 'tuned' Site 721 susceptibility record to generate detailed age-depth relations for the 2.7–2.5 Myr interval, we converted the terrigenous concentrations to accumulation rates²³. Figure 4c shows the strong positive correlation between susceptibility and terrigenous accumulation rate, indicating that the susceptibility variations are reflecting variations in terrigenous supply rather than biogenic dilution; by contrast, the correlation between susceptibility and biogenic accumulation rate was poor. A study of late Pleistocene terrigenous accumulation on the Owen Ridge¹⁶ supports this conclusion.

The strong relationship between susceptibility and the concentration and accumulation rate of the terrigenous fraction, and the occurrence within this fraction of minerals indicative of an aeolian source²³, leads us to conclude that the Site 721 susceptibility record is a direct expression of variations in the wind transport of terrigenous material by the Asian monsoon. The interpretation of the Site 661 susceptibility record as a direct indicator of variations in terrigenous deposition is less certain. Fluctuations of deep water from Northern and Southern Hemisphere sources across the site, possibly at orbital periodicities, could have influenced its susceptibility record through variable calcite dissolution.

The predominance of 23- and 19-kyr variance in the susceptibility records before 2.4 Myr indicates that terrigenous deposition from the Asian, and possibly the African, summer monsoons was largely modulated by summer-season insolation variations due to precession. Atmospheric GCM experiments have demonstrated that monsoonal circulation is extremely responsive to precessional variations in local summer insolation, and that the response is approximately linear¹⁴. The marked increase in variance at the 41-kyr periodicity at ~2.4 Myr coincides with the initiation of major Northern Hemisphere glaciation²⁵, and GCM results and geological evidence indicate that this may reflect ice-sheet effects on the monsoon climate or the dust source regions. The increase in power at the 41-kyr periodicity is particularly significant because the late Pliocene marine $\delta^{18}\text{O}$ record varies almost purely at this periodicity²⁶. GCM experiments have not unambiguously identified any direct effects of the large high-latitude Laurentide and Fennoscandian ice sheets on low-latitude hydrology²⁷, although there is geological evidence^{10,28–30} indicating that African and Asian source areas may have experienced aridification during the last glacial maximum. Hence, the increase in 41-kyr power after 2.4 Myr may be linked to changes in the monsoon intensity or source-area climate due to the coeval expansion of the Eurasian or North American ice sheets, or both, which varied predominantly at this periodicity.

Our results clearly demonstrate the value of whole-core susceptibility logging for identifying potentially important time intervals deserving further investigation. Parallel mineralogical and grain-size studies will help to evaluate more fully the significance of susceptibility as an indicator of source-area climate and terrigenous deposition at both sites. More importantly, the integration of these data with on-going isotope and biotic assemblage studies will lead to a better understanding of the climatic origin of the 2.4-Myr shift and its relevance to other components of climate. □

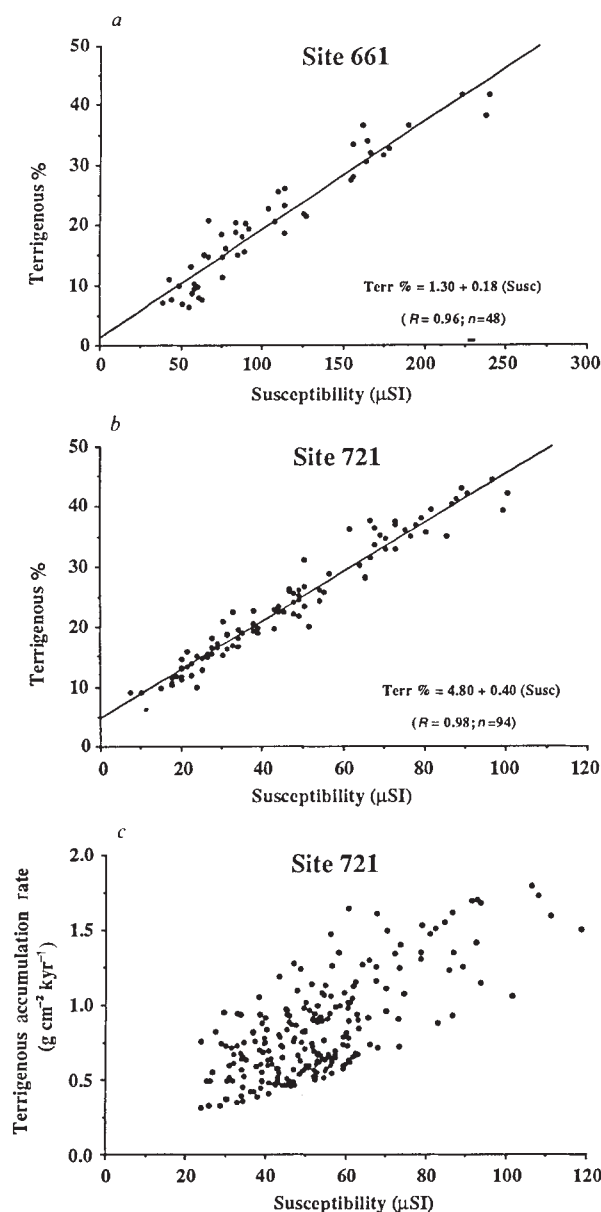


FIG. 4 a, b, Correlation between susceptibility and per cent terrigenous sediment for the Site 661 and Site 721 sediments. The terrigenous per cent values were calculated after sequentially removing calcium carbonate, opal and organic carbon components^{23,24}. c, Correlation between susceptibility and terrigenous accumulation rate for the 2.5–2.7-Myr interval of Site 721. Because the susceptibility is highly coherent with orbital precession over this interval (see Fig. 3), detailed accumulation-rate data were calculated by phase-locking ('tuning') the raw susceptibility record to precession. The resulting accumulation-rate time series was combined with the relation shown in b to calculate the separate biogenic and terrigenous accumulation-rate data. The strong positive correlation indicates that susceptibility at Site 721 is reflecting variations in terrigenous accumulation, not biogenic dilution. The susceptibility–biogenic accumulation-rate plot, for which the correlation is poor, is not shown.

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Thermal entrainment by deflected mantle plumes

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HOTSPOTS are thought to result from deep mantle plumes, perhaps representing an ancient geochemical source isolated from the uppermost mantle. As they rise through the mantle, thermal plumes entrain surrounding material by thermal conduction, and small plumes or narrow plume conduits may consist of only a small fraction of original source material. Here we present new experimental results which show this effect dramatically for plume conduits that are deflected horizontally by shear flow in the mantle beneath lithospheric plates. Thermally entrained material can become concentrated at the centre of a plume (or surface hotspot trace) with original source material in the periphery. Also, the diapiric instability observed in chemical plume conduits is suppressed by thermal entrainment. Varying degrees of geochemical enrichment observed at hotspots such as the Galapagos Islands may result from thermal entrainment, implying that mantle plumes are driven by thermal (rather than compositional) buoyancy.

If volcanic hotspots such as Hawaii are caused by mantle plumes^{1,2}, then the nature of these buoyant plumes is constrained by some striking (although not necessarily consistent) features of hotspots. Hotspot plumes must (1) represent a deep mantle source that is relatively decoupled from lithospheric plate motions, (2) transport material that is geochemically enriched relative to the depleted upper mantle, and (3) represent a horizontal scale length (~10–100 km) of convection that is much smaller than that associated with plate motions.

Although it is not clear from observations whether mantle plumes are driven primarily by thermal or chemical buoyancy, a thermal origin at the core–mantle boundary is commonly assumed. Broad-scale, two-dimensional (sheetlike) thermal plumes have been modelled in many theoretical and experi-

mental convection studies (refs 3–5, for example) in which lower-boundary-layer instabilities develop in response to basal heating. Experimental studies of narrow isolated plume conduits^{6–10} have used chemically buoyant (non diffusive) plume material. Plume conduits exhibit a fascinating variety of behaviour including solitary conduit waves¹⁰ and diapiric instability in horizontally deflected conduits^{7,8}. Thermally buoyant conduits have not been studied experimentally, however, and thermal diffusion may be expected to dominate the dynamics of very narrow plumes. (Diffusion effects are not expected to be important in compositionally buoyant mantle plumes, because chemical diffusion rates in the mantle are generally thought to be much smaller than thermal diffusion rates.) Here we present results from preliminary experiments that demonstrate the important effects of thermal entrainment for plumes deflected horizontally by mantle shear flow beneath the lithospheric plates. These results are particularly applicable to the mixing of enriched and depleted mantle reservoirs by thermal plumes.

The thermal entrainment of surrounding material by rising hot diapirs has been demonstrated by Griffiths^{11,12}. Figure 1 illustrates this process in which a boundary layer of heated surrounding material becomes entrained, because of its own thermal buoyancy, into the rising diapir. As the diapir rises, a torus or smoke ring of original source material forms, with entrained material forming the central core of the diapir for initial diapir Rayleigh numbers larger than about 12 (ref. 11). We show that this process also occurs in a horizontally deflected thermal plume as the plume conduit rises through the cold surrounding material. This can be envisioned by imagining that Fig. 1 represents not a cross-section of a spherical diapir, but instead a view along the axis of a cylindrical conduit that is rising through a fluid (see refs 13, 14). The superposition of fluid flow through the conduit and the bifurcated flow pattern shown in Fig. 1 will give rise to paired, helical particle paths as material ascends through the plume conduit, with thermal entrainment of heated surrounding material into the centre of the plume.

We observed this phenomenon using a simple experimental apparatus described in detail elsewhere¹⁵. A cylindrical Plexiglas tank (60 cm in diameter and 10 cm high) is filled with glycerol and fitted with a lid which is rotated slowly by an electrical motor, creating a simple (circular) shear flow in the tank beneath the lid or plate. The plume is fed gravitationally through a

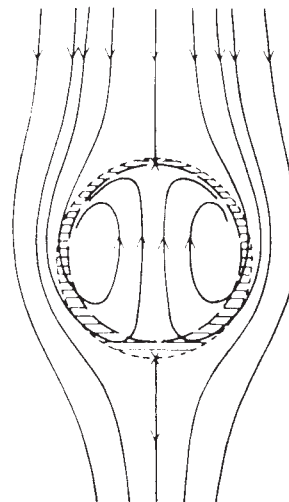


FIG. 1 Paths of fluid particles outside a hot, rising diapir¹¹. Those particles that approach close to the diapir are heated by diffusion in the thin thermal boundary layer (hatched area), become buoyant, and are incorporated into the enlarging spherical vortex. In other words, they become entrained. Alternatively, this illustration can be taken as the view along the axis of a cylindrical plume conduit as in the experiments presented here.

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control valve from an elevated reservoir of glycerol containing a small amount of red dye. The plume source is a small hole (0.2-cm diameter) in the base of the tank, 16 cm from the lid rotation axis. The red glycerol is heated before it enters the tank by heating tape wrapped around the copper inlet pipe. The temperature of the thermally buoyant plume material is monitored by a small thermocouple just below the inlet hole.

The photographs of Fig. 2*a, b* show side and top views of a horizontally deflected plume that exhibits strong thermal entrainment. The red dye (dark material) represents the original plume material injected at the base of the tank, and it forms two stripes along the outside of the plume as seen in top view. The central core of the plume consists of entrained material. The plume remains largely a circular cylindrical conduit, expanding as it rises in the tank. The relevant experimental parameters are: tank-fluid kinematic viscosity $\nu_t = 1.5 \text{ cm}^2 \text{ s}^{-1}$, upper-plate velocity above the plume source $u_0 = 0.23 \text{ cm s}^{-1}$, temperature contrast between plume source and tank fluid $\Delta T = 50^\circ \text{C}$, density contrast $\Delta \rho = 0.018 \text{ g cm}^{-3}$. (The glycerol in this experiment absorbed some water from the air so that its viscosity was about a factor of four less than that of pure glycerol.) In the case shown, the Prandtl number is sufficiently large ($\text{Pr} = 320$) and the Reynolds number (for the rise of the conduit through the tank fluid) sufficiently small ($\text{Re} = 0.01$) to assure dynamical similarity to creeping flow in the mantle. The initial plume diameter is 0.3 cm and it expanded to $>1.0 \text{ cm}$ just beneath the tank lid. (We note that thermal expansion of plumes occurs whether or not there is significant horizontal deflection¹⁵.) This represents about a factor of ten increase in plume cross-sectional area, giving an order of magnitude decrease in average temperature contrast across the plume. The half-angle expansion ratio of the conduit¹¹ is approximately constant at $\epsilon = 0.049$. (ϵ is one-half the increase in plume diameter per unit plume-rise distance.) The original plume material accounts for only about

one-tenth of the thermally expanded plume volume near the tank lid.

We can scale this experiment to plumes in the Earth's mantle by defining a conduit Rayleigh number $\text{Ra} = g\Delta\rho V/\kappa\nu_t$, where g is the gravitational acceleration and κ is the thermal diffusivity of the tank fluid. The Rayleigh number is simply a dimensionless thermal buoyancy, and it is not intended to represent the vigour of mantle convection. V is defined¹³ as an effective 'conduit element' volume based on the initial conduit diameter d so that $V = 2.9\pi d^3/8$. (The factor $2.9\pi/8$ is somewhat arbitrary¹³ and of no consequence with respect to the following scaling arguments.) Thus, the conduit Rayleigh number for the plume of Fig. 2*a, b* is $\text{Ra} = 24$. This can be scaled to the mantle as follows: for a plume of reasonable density contrast 0.02 g cm^{-3} ($\Delta T \approx 200^\circ \text{C}$), diameter 50 km and thermal diffusivity $\kappa = 0.01 \text{ cm}^2 \text{ s}^{-1}$, and for a mantle viscosity of 10^{22} P , we obtain a similar Rayleigh number $\text{Ra} = 29$. The plume-conduit Rayleigh number increases with the cube of the plume diameter and decreases with the viscosity of the surrounding fluid, and neither of these parameters is well constrained for mantle plumes. The experimental results shown are applicable, for example, for mantle-plume diameters of $\sim 5\text{--}80 \text{ km}$ with background mantle viscosities of $\sim 10^{19}\text{--}10^{23} \text{ P}$, respectively. Clearly, the range of conduit Rayleigh numbers in our experiments may apply to real mantle plumes.

Another important parameter is the upper-plate velocity, which is $u_0 = 0.23 \text{ cm s}^{-1}$ here. This can be scaled to lithospheric plate or mantle shear-flow velocity using the Peclet number (dimensionless velocity). The Peclet number is defined as $\text{Pe} = u_0 L/\kappa$, where the appropriate scale length is the tank fluid depth $L = 10 \text{ cm}$. For the experiments shown, $\text{Pe} = 290$. Scaling to the Earth's mantle depth $L_m = 3,000 \text{ km}$ and using $\kappa = 0.01 \text{ cm}^2 \text{ s}^{-1}$, we obtain the characteristic mantle shear velocity $u_m = 0.32 \text{ cm yr}^{-1}$. This is appropriate either for a very slow litho-

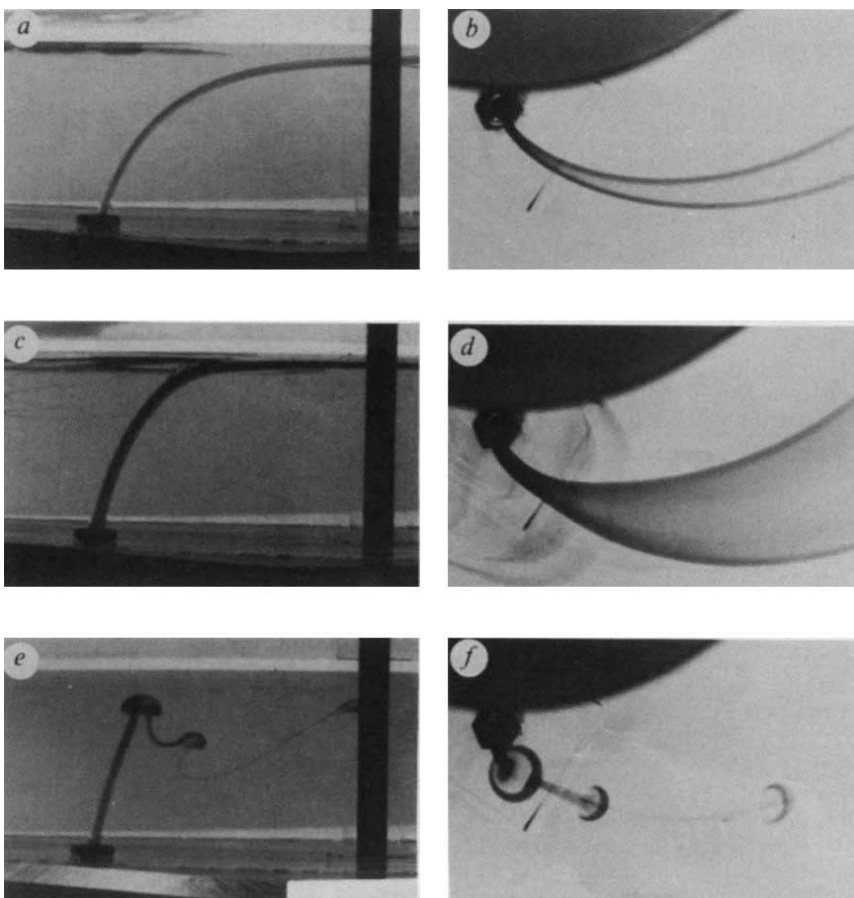


FIG. 2 Side (*a, c, e*) and top (*b, d, f*) views of the plume experiments. In *a, c* and *e* the dark vertical bar on the right-hand side is the lid rotation axis. The 1-cm-thick lid is the light horizontal band just above the point where the red (dark), thermal plume becomes horizontal. In *b, d* and *f* the drive wheel appears dark and unfocused in the upper left-hand corner. The lid rotation is counter-clockwise along the curvature of the dark plume trace. The faint grey cloud above the plume source in *d* is diffused plume material from previous experiments that has been advected around the cylindrical tank.

spheric plate or as a characteristic velocity in the Earth's deep mantle. (The relatively low-viscosity upper mantle, or asthenosphere, beneath the plates indicates deep-mantle flow velocities about an order of magnitude less than typical plate velocities¹⁶.) Larger upper plate or shear-flow velocities, with other parameters constant, will result in more horizontal deflection¹³ and even stronger thermal entrainment effects, especially for higher Rayleigh numbers.

Based on experiments with hot rising diapirs¹¹, we expect significant thermal entrainment for Rayleigh numbers as high as $\sim 10,000$. Figure 2c, d shows a larger plume (higher plume feed rate) under experimental conditions that are otherwise the same as for Fig. 2a, b. The initial plume diameter is 0.6 cm, yielding a higher conduit Rayleigh number of 192. The measured expansion ratio is $\epsilon = 0.027$, which is smaller than for the previous experiment ($Ra = 24$; $\epsilon = 0.049$). This is expected, because a higher Rayleigh number implies a smaller thermal boundary-layer thickness relative to the conduit diameter. The two experiments here indicate a somewhat smaller decrease in ϵ with increasing Rayleigh number than for diapirs¹¹. For the higher-Rayleigh-number experiment in Fig. 2c, d the original plume material constitutes only about 35% of the volume of the plume as it encounters the tank lid.

Returning to Fig. 2a, we note that in this experiment and for other thermal-plume conduits deflected to almost horizontal orientations (data not shown), we never observed any diapiric-conduit instability. Apparently, plumes of modest Rayleigh number are stabilized by thermal entrainment of surrounding material. This is an important observation, because diapiric instability in deflected plume conduits (observed in laboratory models of chemical plumes) has been suggested as the cause for individual volcanic centres along hotspot tracks⁷ and for sea-floor swells⁹. Thermal plumes probably remain stable because of the ever expanding thermal-conduit width and because of the reduced viscosity contrast across the plume. (At a temperature contrast of 50 °C, the plume interior is initially about an order of magnitude less viscous than the surrounding fluid.) A detailed stability analysis, similar to Whitehead's for chemical plumes¹⁷, is thus warranted for thermal plumes.

Figure 2e, f show another new observation. Thermal conduit waves (or solitons) are induced by three sudden increases in conduit feed rate. These three blobs show the thermal entrainment expected¹¹, but the effect of horizontal deflection causes a strongly asymmetrical pattern in the original source material as the blobs rise. If such hot blobs are considered as hotspot sources, then the original (enriched mantle) plume material would be least diluted in the 'downstream' direction of plate motion with the most strongly diluted material toward the 'upstream' side of the volcanic centre.

The most conspicuous evidence for the effects we have discussed is found in the geochemistry of basaltic lavas from the Galapagos hotspot, for which Geist *et al.*¹⁸ have described a puzzling open-horseshoe pattern of strontium isotope enrichment. This pattern is thought to reflect source heterogeneity, because variations in trace-element concentrations, which reflect differences in melting and fractionation processes, are not well correlated with the variations in the isotope ratios. Geist *et al.*¹⁸ proposed that this source heterogeneity resulted from thermal entrainment by a persistent hot diapir beneath the lithosphere, with a depleted (upper mantle) core surrounded by an enriched (lower mantle) torus. The least-enriched basalts are found toward the downstream (east) side of the Galapagos archipelago, however, which is just the opposite pattern shown in our experiments (Fig. 2f).

There are other problems with the single-diapir model for the Galapagos hotspot. First, a solitary diapir would obviously not remain stationary beneath the lithosphere, but would be advected along with the plate motion. This is inconsistent with the fact that the volcanism propagates westward relative to the overlying oceanic plate at the expected rate (~ 5 cm yr⁻¹) for a

fixed mantle plume. The second problem is that the continuity of the Cocos and Carnegie Ridges (twin hotspot tracks formed while the Galapagos spreading centre coincided with the Galapagos hotspot) requires long-lived continuity of the Galapagos plume before ~ 5 Myr.

We suggest an alternative model which might better explain the Galapagos isotope data. Thermal entrainment by a continuous, horizontally deflected plume would result in a spreading V pattern (see Fig. 2b, d), with the least-enriched (upper mantle) source material toward the downstream side of the hotspot track (as observed). Scaling from the experiment in Fig. 2d to the ~ 150 -km-wide plume top needed to explain the width of the Galapagos platform, we find a comparable conduit Rayleigh number of ~ 200 for an upper-mantle viscosity of 10^{22} P and an original plume excess temperature of ~ 250 °C. Scaling to the upper-mantle depth of 600 km, a comparable Peclet number of ~ 300 requires an average upper-mantle shear velocity of ~ 1 -2 cm yr⁻¹. As in Fig. 2d, such a plume would consist of only about 35% original source material, with a final temperature excess beneath the lithosphere of ~ 100 °C. The parameters we have assumed are not well constrained, and we emphasize that our model is speculative. A major field effort is presently underway in the Galapagos to test these ideas, and a more complete set of laboratory experiments will be reported elsewhere.

Our experimental parameters emphasize the true plume width and maximize the degree of dilution or entrainment. Differing degrees of thermal entrainment (or horizontal deflection) may explain some of the variations in the degree of geochemical enrichment observed for different hotspots¹⁹. Application of these ideas to geochemical data from hotspot tracks such as the Galapagos may help determine whether or not mantle plumes are driven primarily by thermal or compositional buoyancy. □

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Gravity changes as a precursor to volcanic eruption at Poás volcano, Costa Rica

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MONITORING of gravity changes at active volcanoes has considerable potential for understanding magma-chamber dynamics and for detecting eruptive precursors. The technique essentially aims to identify changes in sub-surface mass or density corresponding to departures from the free-air gradient (FAG) or Bouguer-

corrected (terrain-corrected) FAG (BCFAG), respectively^{1,2}. Such gravity changes, of the order of 10–100 μgal , have been reported previously through eruptive periods^{3–6}. Here we present a ten-year data set measured at Poás volcano, Costa Rica, in which gravity increases in 1987–1989, relative to both the FAG and the BCFAG, preceded the ash-cloud eruptions of April–May 1989. These results constitute the first unambiguous detection of gravity changes as a precursor to eruption, and we interpret them in terms of the intrusion of an ascending, relatively dense magma body.

The last major eruption at Poás was in 1953, when phreatic activity culminated in the growth of a 30-m-high pyroclastic cone and the formation of an inner crater, both within the old summit crater. Since then, a hot crater lake has absorbed any fluctuations in heat output⁷ from the magma feeder pipe by changing in volume, temperature and evaporation rate. Micro-gravity monitoring at Poás began in 1979 and was intensified to include more stations and some elevation control in 1985 (refs 8, 9). Compared with a reference point 2 km south of the active crater, we observed no significant gravity change at the three crater rim or eight flank stations between 1979 and 1983, although intensive monitoring over a 43-day period in 1983 did reveal cyclic variations⁸. We interpreted this phenomenon, observed again in 1985 during more detailed monitoring of sixteen crater stations, in terms of cyclic variations in the degree

of vesiculation within the summit magma feeder pipe⁹. Since 1985, we have observed unidirectional gravity changes at crater stations on and around the 1953 cone. Relative elevation measurements, using a theodolite with an infrared electronic distance-measuring device yielding a precision of ~ 2 cm, have been made since 1987 so that we could examine the possibility of sub-surface mass changes.

To observe long-term gravity changes, we average the data for each period of observation (typically several days), thus smoothing out any short-period cyclic variations. Uncertainties (which we determine by the reproducibility of difference measurements) are, at worst, up to 40 μgal for data obtained before March 1987, and are < 25 μgal after this date because of the use of electronic readout and many more repeat readings¹⁰. Although there are a few erratic readings in the data set, nevertheless the mean difference between the two instruments is only 28 ± 26 μgal , similar to the uncertainty analysis above (and in ref. 10). The gravity variations relative to the reference point observed between 1979 and 1989 at crater-rim stations (R2A and R7, for example) are irregular and do not show a clear trend (Fig. 1). At crater-bottom stations E1, E3 and D1 (adjacent to the 1953 cone), however, relative gravity has clearly increased since March 1987. Indeed, total changes at the three stations on the 1953 pyroclastic cone (Fig. 1 inset) have been of the order of 200 μgal between 1985 and 1989. On the north side of the crater lake, only station D3 has experienced unambiguous decreases in the relative gravity from 1985 onwards. Because the gravity changes at stations E5–D1 (Fig. 1) are more than several times the size of the uncertainties involved, and two Lacoste and Romberg meters have been used (since 1987), the increases are undoubtedly genuine.

To evaluate the Bouguer-corrected free-air effect on gravity we assume a typical density. Measurements of surface rocks in the crater area yield densities of ~ 2.67 Mg m^{-3} (for lavas) falling to ~ 1.17 Mg m^{-3} for pyroclastic ashes, giving an estimated average of ~ 2.2 Mg m^{-3} for the shallow and surface materials. For a given elevation change, assuming the usual free-air gradient and no density change, gravity will vary according to

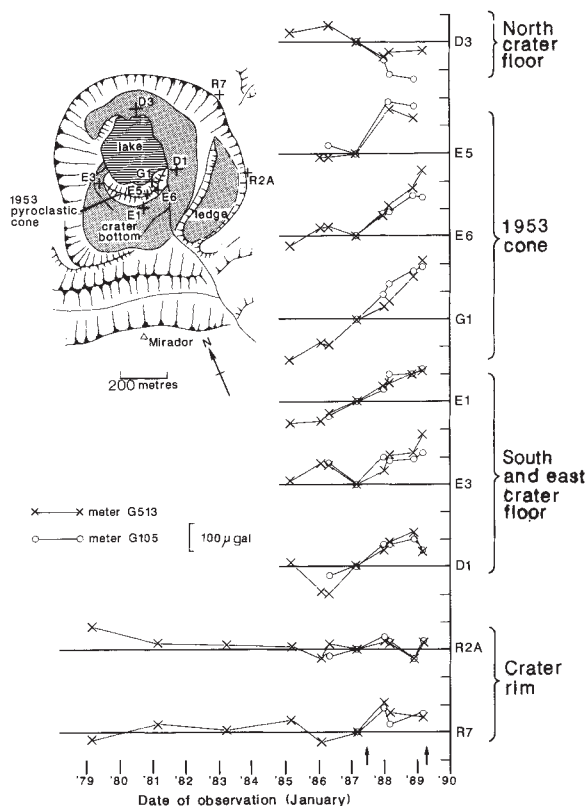


FIG. 1 Gravity variations using Lacoste and Romberg gravity meter G513 and (since 1987) G105 are expressed relative to the value at a reference point on the volcano flank for two crater-rim and seven crater-bottom stations with good gravity and elevation control. Each data point represents the average value obtained during 3–10 survey days. For details of errors, see text. The two arrows at the bottom of the figure indicate periods of dramatic change in surface activity. Note that if crater-bottom (or rim) gravity data were affected by the recent decrease in lake level, a relative decrease in gravity would be observed (as at D3, which seems to be remote from the centre-of-mass increase). Because all stations except D3 experience a gravity increase, the effect of the falling lake and water table is either negligible or at least small—except on the northern lake side—compared with another opposite effect causing a gravity increase. The inset shows station locations.

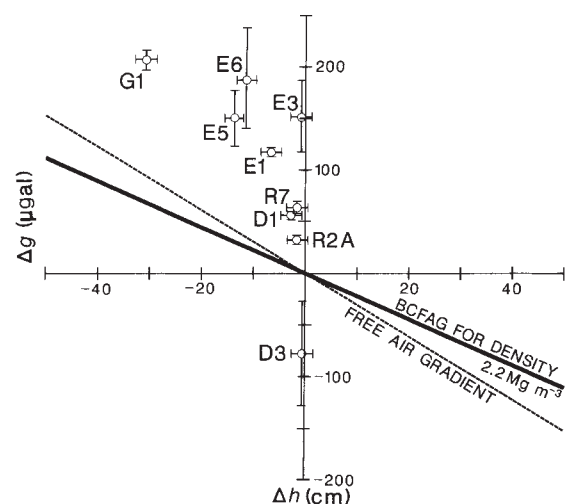


FIG. 2 Plot of the change in gravity against the change in elevation with the theoretical FAG and BCFAG marked for reference^{1,2}. The mean gravity change as recorded by the two instruments is plotted with error bars reflecting the difference between the two meters for the 1987–1989 comparison (see Fig. 1, where the two meters differ by 28 ± 26 μgal). The stations read most frequently, which are G1, D1, E1 and R2A, have the smallest error bars on this basis. Errors in elevation differences are estimated as ± 2 cm. An overall gravity increase is observed between March 1987 and March 1989 at all crater stations, except D3, even after account is taken of deflation, because all points plot above the FAG and BCFAG lines. The residual gravity increase over this period beneath stations E1, E3, E5, E6 and G1 is 100–150 μgal ; see text for interpretation of the corresponding shallow mass increase.

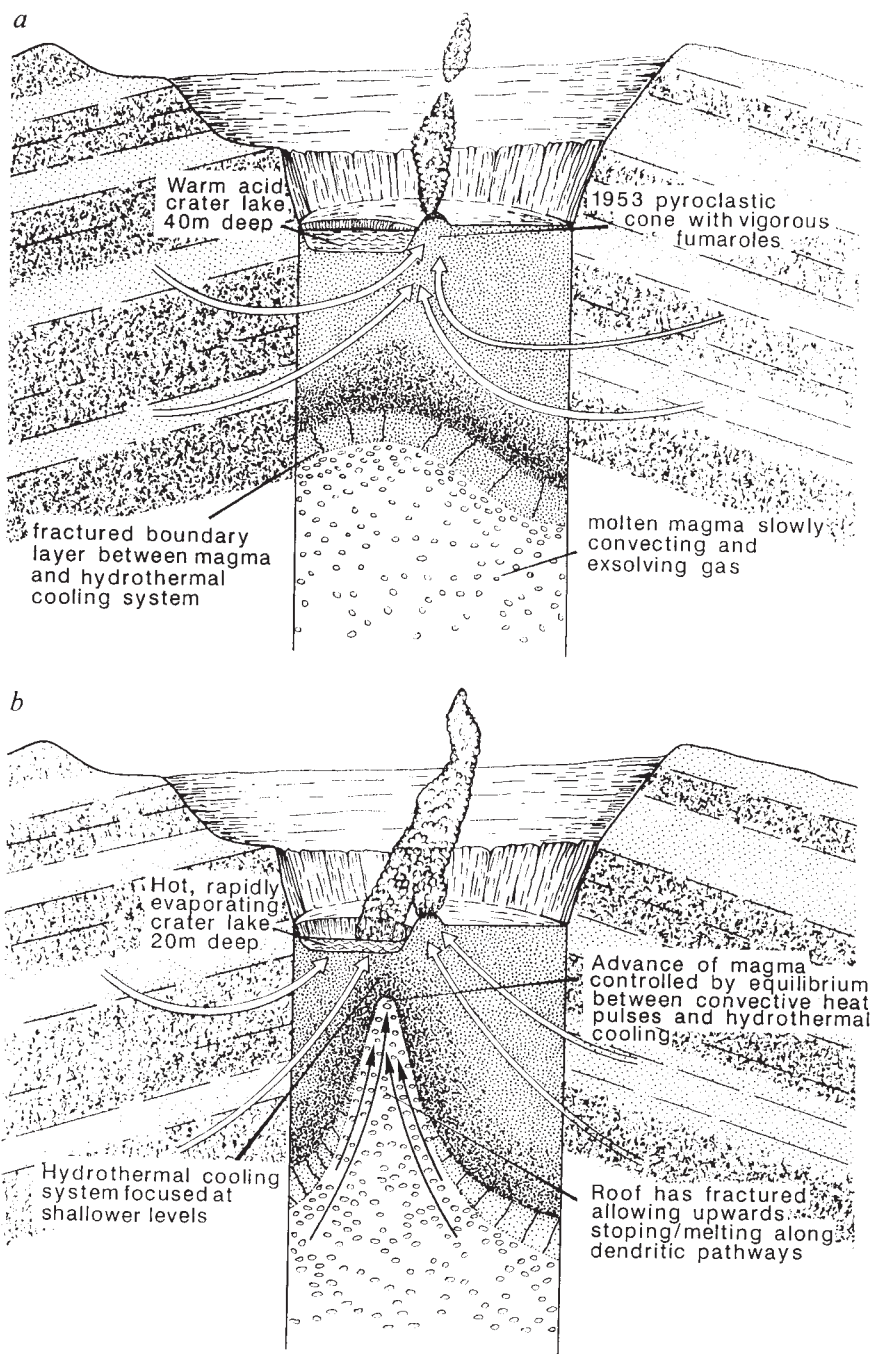
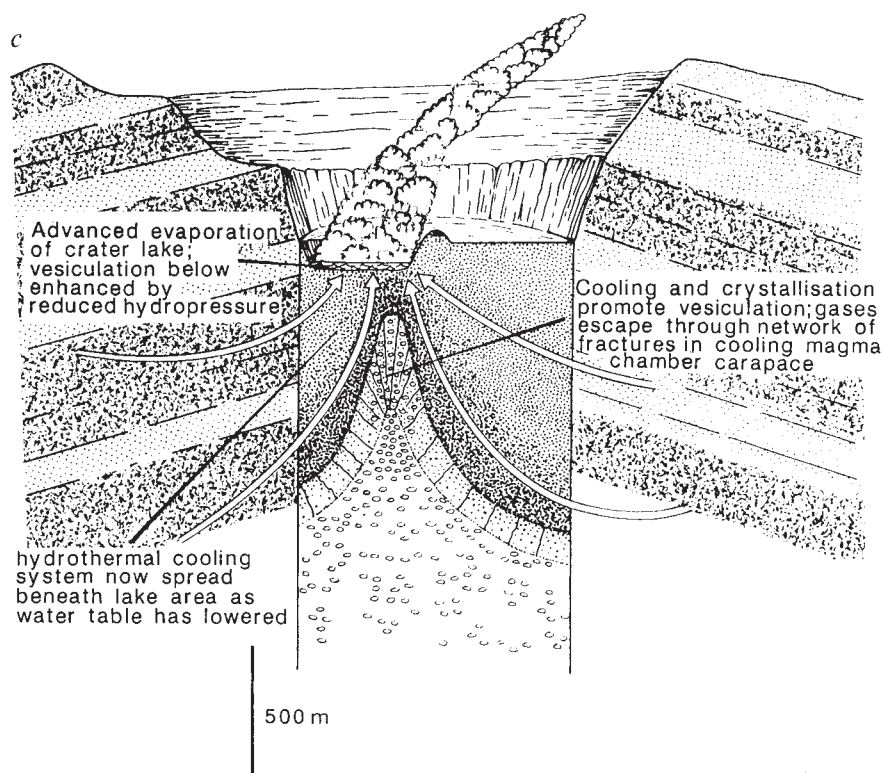


FIG. 3 Schematic north-south cross-sections of Poás volcano summit and postulated evolution (*a-c*) of the magma chamber and its upper boundary layer. Within this layer, heat is removed by the hydrothermal steam-water system from the frozen, fractured cap of the partially molten magma body, initially at ~500 m depth. *a* shows the steady-state 1985 model developed in refs 7 and 16; by 1987 (*b*) we propose that the boundary layer had fractured extensively, allowing fingers of magma to ascend to a new equilibrium level where their upwards progress was halted by the increasingly efficient cooling system; in late 1988 (*c*), as pressure on the cooling magma system reduced and crystallization advanced, the collection of volcanic gases within the magma and their discharge through an extended fractured boundary layer is envisaged.

the BCFAG. The predicted BCFAG for this density is $-216 \mu\text{gal m}^{-1}$ and we attribute (to a first approximation) observations that fall away from this line to sub-surface density changes¹⁻⁶. This simple model requires change to occur only along an extensive horizontal slab; more complicated regions of dilatation/contraction do not follow a linear $\Delta g-\Delta h$ relationship². We present data for March 1987-March 1989 in Fig. 2; although there is some scatter, it is clear that overall subsidence of the crater rim and bottom region (maximum of 31 cm, measured relative to the Mirador benchmark shown in Fig. 1 inset) was accompanied by an 'excess' gravity increase. A net gravity increase above the BCFAG during a period of elevation decrease reflects an overall sub-surface density increase and a net gravity increase above the FAG reflects an overall mass increase. The excess gravity increase above both the BCFAG and FAG is smallest on the crater rim and largest on the pyroclastic cone, which is also the region of maximum deflation (especially at station G1). For stations E1, E3, E5, E6 and G1

the excess gravity increase above the FAG is 100-150 μgal (Fig. 2).

Records of historical activity at Poás^{11,12} show that the recent geysering and disappearance of the lake, culminating in ash eruptions (as in 1989) are following a similar pattern to the rather poorly documented 1828, 1834, 1910 and 1953 eruptions. A warm (~40 °C) acidic crater lake remains apparently quite stable for a number of years, then shrinks dramatically and disappears during phreatic activity, which often culminates in a relatively minor ash-pyroclastic eruption. Before the present activity the Poás crater lake had maintained dynamic equilibrium since the late 1970s (refs 7, 13), however the lake level began to fall slowly in 1982 and by March 1989 it had fallen by a total of 40 m, 32 m of which occurred between March 1987 and March 1989 (refs 7, 14). Changes in the style of surface activity are marked on Fig. 1 at June 1987 when geyser eruptions began¹⁴ and April 1989 when a 2-km-high ash cloud (reworked material) was erupted periodically until the crater lake was



re-established in mid-May. Interestingly, we observe the most significant gravity changes at stations on the 1953 cone both before 1987 and during the 1987–1989 period. Elevation data are not available before 1987, but gravity data for most crater stations show a similar trend from 1985 onwards, so assuming that elevation changes were no greater than those since 1987, the magnitude of the excess gravity increase over the four-year 1985–1989 period is estimated at $\sim 200 \mu\text{gal}$ over an area of $\sim 10^4 \text{ m}^2$. By applying Gauss' theorem and assuming the new mass has a density 2.67 Mg m^{-3} (the same as the erupted lava) we calculate a total sub-surface mass increase of $\sim 10^8 \text{ kg}$.

An overall density increase beneath the crater area of volcanoes like Poás is usually interpreted^{1,3} either in terms of magma devolatilisation, or the collapse of voids, or the ascent of relatively dense magma into low-density agglomerates and ashes. In view of the 1987–1989 deflation, void collapse is a possibility, but because the power output at Poás almost doubled between 1985 and 1988 (ref. 7), we prefer a model involving upwards ascent of magma. As the 1985–1987 gravity increase is only observed at stations on and close to the 1953 cone, and thereafter the effect is largest at these stations, the depth of the causative intrusion using a half-width approximation must be shallow, probably $< 200 \text{ m}$. The intrusion is not necessarily directly beneath the cone, because gravity increases more to the north (that is, from E1 to E6 to G1); it may be centred on the fault at the north side of the cone or even further north beneath the lake (where we have no data). Given the steady-state starting situation in Fig. 3a (refs 7–9), we suggest that a small dendritic intrusion rose (Fig. 3b) from a region of partial melt $\sim 500 \text{ m}$ beneath the crater through cracking of the magma-chamber roof zone¹⁵, perhaps because hydrothermal cooling was unable to extract heat at the rate being supplied from below⁷. As the magma advanced it drove off ground waters producing elevated fumarole temperatures (500°C measured on the cone in 1985). As the lake level fell because of more rapid evaporation, the water table would become lower (which may account for the gravity decreases observed at station D3) and eventually the fumaroles on the cone would have cooled and almost disappeared as heat output switched to the crater lake (the bottom

of which is some 30 m below the base of the cone; Fig. 3c). As the cone cooled from $\sim 500^\circ\text{C}$ (1985) to $\sim 100^\circ\text{C}$ (1988), it would be expected to contract. The cone consists of vesiculated, blocky and altered andesite lava, with a thermal expansivity of $\sim 5 \times 10^{-5} ^\circ\text{C}^{-1}$, so a drop of 400°C could easily result in 1% contraction of the 30-m -high cone (note the 31-cm deflation at station G1, Fig. 2). Deflation would also accompany the fall in water vapour pressure within the edifice as the water table fell below the base of the cone to its early 1989 position just above the lake bottom (Fig. 3c).

This is the first unambiguous record of gravity changes occurring before an eruption (in this case, the April–May 1989 ash cloud); it highlights the potential of microgravity monitoring as a predictive technique in the surveillance of active volcanoes. The power output at Poás is calculated to have increased from $\sim 160 \pm 50 \text{ MW}$ to $\sim 265 \pm 100 \text{ MW}$ between 1985 and 1988 (ref. 7) and this is consistent with the injection of shallow magma beneath the crater lake (Fig. 3b). The possibility of a major pyroclastic eruption involving juvenile material, resulting from the advanced buildup of magmatic gas pressure⁸ in the area of the pyroclastic cone and lake, cannot be ruled out. $\square$

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A helium isotope transect along the Indonesian archipelago

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HELIUM isotope studies^{1,2} have identified the Banda arc section of the Indonesian archipelago as the only circum-Pacific volcanic arc with ³He/⁴He ratios significantly lower than the common ratio in mid-ocean-ridge basalts (MORB). In eleven of twelve circum-Pacific arcs, MORB-like ³He/⁴He ratios show that helium in these volcanic terrains is dominantly primordial helium from the mantle wedge above the sinking slab, rather than radiogenic helium from subducting oceanic crust or sediments. Only in the Banda arc have significantly lower ratios been observed, corresponding to as much as 80% radiogenic helium from subducting continental material². We now report ³He/⁴He measurements along a 1,800-km section of the Sunda arc, a region of underthrust oceanic lithosphere, showing MORB-like ratios from western Java to a surprisingly sharp transition zone centred on Lomblen Island, where the low ratios of the Banda arc begin. Our results confirm the prediction² that Sunda arc volcanoes should have MORB-like ³He/⁴He ratios, with a well-defined transition to the much lower Banda arc ratios near Lewotolo volcano, and demonstrate a remarkable linkage of helium isotope variations with tectonic processes not readily discernible with trace elements or other isotopes.

The Indonesian arc system is a study in contrasting tectonics³⁻⁵. In the Sunda arc on the west, oceanic lithosphere of the Austral-Indian plate is subducting northward along the Java Trench under the continental margin of the Eurasian plate. On the east the contiguous Banda arc is a reciprocal system with northward subduction of the Australian continental margin, also on the Austral-Indian plate, under the Banda Sea. Both arcs include typical arc volcanics and high-potassium calc-alkaline lavas⁶⁻⁸. The Sunda arc however, along 2,000 km from western Java to Lomblen, is punctuated at ~650-km intervals by leucite-bearing potassium-rich volcanoes⁹, such as Muriah on Java¹⁰, Sangenges and Soromundi on Sumbawa¹¹ and Batu Tara north of Flores⁹. Strontium and neodymium isotope data⁹⁻¹⁶ are the primary discriminants for the two arcs: Sunda arc lavas lie within the mantle-array region defined by other island arcs, whereas Banda arc lavas are displaced toward more radiogenic Sr and Nd ratios, indicating mixing with sialic crustal material¹⁷. The isotope data and tectonic interpretation are thus consistent with one another and indicate that the Banda arc is the only volcanic island arc and continent collision zone in which underthrusting of continental crust is occurring.

We measured the ³He/⁴He ratios of volcanic gases from nine sites at five active volcanoes on Java, Flores and Lomblen: helium in phenocrysts from recent lavas was measured at three other volcanoes on Bali, Sumbawa and Batu Tara (Komba). In Fig. 1 and Table 1 we give the ³He/⁴He ratios at each site; data from our previous study² of Banda arc volcanoes are also included. Lewotolo volcano was resampled (at different sites) with identical results. The most important results show that the Sunda arc volcanoes along a 1,500-km section from western Java to central Flores (Tangkuban Parahu, Merapi, Batur, Sangeang Api and Keli Mutu) have ³He/⁴He ratios of $R/R_A = 5.7-8.2$ (where R_A is the ³He/⁴He ratio of air), almost exactly the range in the circum-Pacific arc systems studied previously². Compared to the mean MORB ratio ($R/R_A = 8 \pm 1$) and radiogenic or 'crustal' He ($R/R_A < 0.02$), these volcanic ratios correspond to a mantle (MORB) helium fraction of 70-100%. The Java-Bali-Sumbawa-Flores ³He/⁴He ratios are thus very different from the Pantar and Banda arc ratios ($R/R_A = 1.1-1.7$) in which the helium is in every case more than 80% of radiogenic origin. Because the Banda arc is unique among volcanic-arc terrains in emitting predominantly radiogenic helium, and is unique in having collided with, and been underthrust by, continental crustal material, it is evident that the Banda arc helium is derived from the subducting Australian crustal material.

Between the low R/R_A volcano Sirung on the island of Pantar and the first active volcano of the Banda arc, Damar, a 500-km inactive segment has been interposed by completion of the suture between the Banda arc and the Australian continental margin and cessation of active underthrusting^{3,4}. The transition from mantle to radiogenic helium emission is not in the Banda arc itself, nor is it across the volcanic quiet segment: the actual ³He/⁴He transition zone is the narrow 200-km section between Egon volcano on the west, at the narrowest width of eastern Flores, and Sirung volcano on the east, at the southern tip of Pantar¹⁸. These two volcanoes have the lowest ³He/⁴He ratios in the arcs ($R/R_A = 1.25$ and ~ 1.1), but between them, at nearly the midpoint of the transition zone, Lewotolo volcano within the arc and Batu Tara 53 km to the north in the Banda Sea have identical elevated ³He/⁴He ratios of 3.4 and 3.6 R_A . The ³He/⁴He structure of this zone is thus complex and deserves further study.

In Fig. 2 we compare the ⁸⁷Sr/⁸⁶Sr profile (see refs 6, 10 and 19) and the ³He/⁴He ratios along the 2,800-km section from west Java to Serua at the eastern extremity of the Banda arc. The sharp drop in ³He/⁴He ratio at the transition zone (6.0 R_A to 1.25 R_A in only 70 km from Keli Mutu to Egon) is in marked contrast to the approximately linear increase in ⁸⁷Sr/⁸⁶Sr over the ~750-km section from Bali to Egon, and even more so for the 1,500-km increase from Bali to Damar. That is to say, the along-strike transition from oceanic lithosphere to continental crustal material on the downgoing Austral-Indian plate, from the ³He measurements, seems to be extremely sharp. Katili and

FIG. 1 Helium isotope ratios (relative to atmospheric helium) in volcanic gases (▲) and lavas (■) in the Indonesian volcanic arcs. From west to east in the Sunda arc the volcanoes are Tangkuban Parahu and Merapi on Java, Batur on Bali, Sangeang Api on Sumbawa, Keli Mutu and Egon on Flores, Lewotolo on Lomblen, Batu Tara (a potassium-rich leucitic volcano) in the Banda Sea 53 km north of Lewotolo, and Sirung on Pantar. Between the active Sunda and Banda volcanoes is a 500-km volcanoseismic 'quiet sector' extending from Pantar to Damar. Dashed lines correspond to proposed faults in the Savu Basin (stippled): the eastern fault runs east of Pantar; the western fault is assumed to run east of Flores¹⁹ or west of Pantar^{24,25}. Volcano names follow the usage of Simkin *et al.*²⁸.

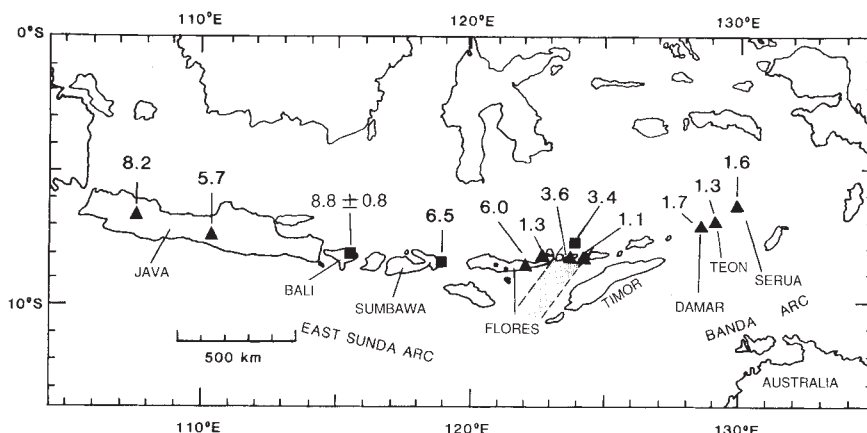


TABLE 1 Composition of Indonesian volcanic gases

Location	Sample type*	T(°C)	R/R _A	He/Ne (He/Ne) _A	R _c /R _A	N ₂ /Ar	He/NC† (p.p.m.)
Java							
Tangkuban Parahu	as	57	8.16	991	8.17	856	450
Merapi Craters							
Gendol	sf	460	4.54	4.0	5.70		
Gendol	sf	460	4.50	4.7	5.43	85.0	27.3
Woro	sf	500	3.49	2.2	5.62	84.9	14.0
Woro	sf	500	3.40	2.1	5.49		
Bali							
Batur	ol				8.8 ± 0.8		[He] = 2.1 × 10 ⁻⁹ cm ³ g ⁻¹
Sumbawa							
Sangeang Api	cpx				6.52		[He] = 6.6 × 10 ⁻⁹ cm ³ g ⁻¹
Flores							
Keli Mutu	wa	51	5.92	68.6	6.00	48.9	[He] = 2.3 × 10 ⁻⁶ cm ³ g ⁻¹
Egon I	sf	124	1.27	312	1.27	134	898
Egon II	sf	98	1.23	83.2	1.23	99.4	440
Lomben							
Lewotolo							
Loc.1	sf	140	3.67	2,503	3.67	402	4,194
Loc. 3	sf	300	3.45	185	3.47	161	1,375
Loc. 5	sf	333	2.96	4.0	3.61		
No. 1‡	sf	490	3.62	500	3.62		3,094
No. 2‡	sf	490	3.57	1,1180	3.57		4,163
Batu Tara	cpx				3.44		[He] = 1.5 × 10 ⁻⁹ cm ³ g ⁻¹
Pantar							
Sirung‡	sf	95	1.03	1.7	(1.07)		
Damar‡	ff	135	1.69	600	1.69	239	11,120
‡	sf	170	1.65	3,200	1.65	179	11,600
Teon‡	sf	99	1.31	130	1.31	98.5	1,719
Serua‡	sf	102	1.27	1.7	1.64		

We used a Clarke-type ³He mass spectrometer (GAD) for the ³He/⁴He measurements^{1,2}. Helium was separated from neon on activated charcoal at 37 K and measured relative to the Yellowstone 'Murdering Mudpots' standard ($R/R_A = 16.45$). Neon was also measured and the He/Ne ratios (column 5) were used to correct the measured ratios (column 4), assuming all Ne to be atmospheric. R_c is the corrected ratio and R_A is the ratio in atmospheric helium. Precision of the ³He/⁴He ratios is 1%. The helium was extracted from phenocrysts in recent lavas by vacuum crushing: [He] is the measured He concentration. Blank contribution to ³He was 0.7% (Sangeang Api), 7% (Batur) and 10% (Batu Tara). Only 0.5 g of olivine was available for the Batur analysis: a 10% uncertainty is assigned to the ratio of this sample.

* Sample key: sf, summit fumarole; ff, flank fumarole; as, gas from acid spring; wa, hot spring water sample; ol, cpx, olivine and pyroxene phenocrysts in lavas. Lava samples: Batur, mixture of 1904 and 1974 flows; Sangeang Api, xenolith or cumulate sample 'B4'¹³ (also R. Varne No. 48083) from 1911 lahar on south-west coast; Batu Tara, potassium-rich leucite basanite^{9,16} (R. Varne No. 67130).

† NC, Non-condensable gases. Samples were collected in evacuated 1720-glass flasks (50-ml volume) with vacuum stopcocks. Analyses were made using thermal conductivity gas chromatography, after liquid nitrogen separation of CO₂, H₂S and SO₂ (data to be reported elsewhere). Hydrogen was observed only qualitatively in our system: significant amounts of H₂ were present in the Tangkuban Parahu and Lewotolo samples and traces were seen in the Merapi analyses. Methane was found in gases from Tangkuban Parahu (6.2% of NC), Lewotolo (2.4% of NC) and Damar ff (0.3% of NC).

‡ The ³He and He/Ne data for these samples are from our previous work². (He/NC ratios tabulated for these samples were calculated incorrectly). The ³He/⁴He ratio for Sirung is questionable because of the large amount of air helium in this sample.

Hartono⁵ placed this transition zone "in the eastern part of Flores Island", in close agreement with the position located here by helium isotope ratios.

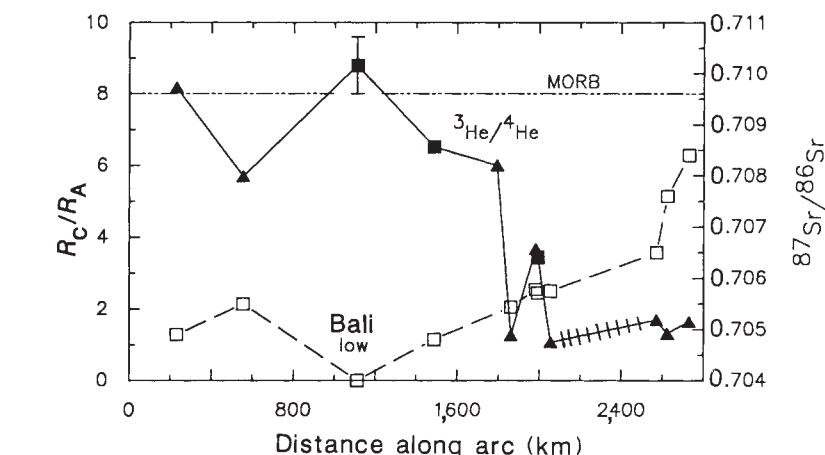
Within the transition zone the ³He structure is marked by higher R/R_A values at Lewotolo and Batu Tara in the centre and more radiogenic ratios at the two volcanoes, Egon and Sirung, that define the edges. Although this pattern is not yet explained there are a number of possibilities. Wheller *et al.*⁹ identified three sectors along the Sunda arc from Java to Lombok that are terminated by the potassium-rich, leucite-bearing volcanoes noted above. These mark the ends of volcanic sectors with progressively increasing K/Si ratios, after which the series begins again in the next sector. Their 'Flores sector' is terminated by the potassium-rich volcano Batu Tara that, with Lewotolo—a high-potassium volcano—marks the centre of the ³He transition zone (Fig. 2). Wheller *et al.* show that K, Si, and ⁸⁷Sr/⁸⁶Sr data for Sunda-Banda lavas require, in addition to ocean mantle and continental crust sources, a third, potassium-rich, component. This component may be the source of the elevated ³He/⁴He ratios in the central part of the ³He transition zone.

An alternative explanation may lie in the relationship between volcanism and tectonics in the Savu Basin, a depression with large negative gravity anomalies which is floored by oceanic

crust and contains the ³He transition zone between the islands of Flores and Pantar. Nishimura *et al.*²⁰ showed that the transition zone is a discontinuity marked by offsets of gravity anomaly, earthquake patterns, submarine morphology, strike of the volcanic arc and chemistry of Quaternary lavas. McBride and Karig²¹ interpreted the gravity data as evidence for the subduction of continental material into the mantle below the basin, and Eva *et al.*²² have proposed that the strike of the subducting slab is parallel to Timor in the eastern part of the basin, resulting in transcurrent faulting such as the Pantar fracture zone deduced from seismic data²³. Silver *et al.*²⁴ and van Bergen *et al.*²⁵ show cross-arc faults both east and west of Pantar in the eastern part of the transition zone, whereas Varekamp *et al.*¹⁹ propose that the Savu Basin is bounded east and west by NNE to SSW transcurrent faults crossing the arc tangent to the east side of Pantar and the eastern end of Flores, almost exactly the arc section outlined by the ³He transition zone. Such cross-arc fault patterns might provide zones for trapping subducted continental material at the boundaries of the Savu Basin while allowing less-contaminated mantle material to erupt in the centre of the basin.

Perhaps the most difficult question posed by our data is the very low ³He/⁴He ratio (1.25 R_A) at Egon Volcano on eastern Flores. Hamilton (ref. 4, Figs 53 and 78) indicates that continen-

FIG. 2 $^3\text{He}/^4\text{He}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios along the Sunda-Banda arcs from the western end of Java. Symbols as in Fig. 1: geographic coordinates are from ref. 18. MORB, $^3\text{He}/^4\text{He}$ ratio ($R/R_A = 8 \pm 1$) in mid-ocean-ridge-basalt helium^{2,27}. Sr isotope data (open squares) are from refs 8–16 (the dashed lines are profiles from refs 6, 10 and 19). The lowest (least radiogenic) $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are in lavas from Bali; eastward from Bali the Sr ratios increase monotonically to the sharp increase in slope at the end of the Banda quiet zone (cross-hatched). The $^3\text{He}/^4\text{He}$ ratios, however, drop very sharply (factor of five) in the 68-km span from Keli Mutu to Egon. The antithetic correlation of Sr and He ratios in the Sunda arc is consistent with simple two-component mixing. Helium data from two sources, fumarole gases and lava phenocrysts, are compared with the Sr isotope ratios. The only detailed comparison so far of such sources is of data from the Mariana arc²: in this area helium from one clinopyroxene and two olivine samples from two seamounts and from a hot spring on Agrigan volcano^{1,2} had identical $^3\text{He}/^4\text{He}$ ratios. But it was also found² that 'flank' fumaroles on large volcanoes such as Makushin tend to have lower $^3\text{He}/^4\text{He}$ ratios than 'summit'



fumaroles: thus caution is necessary when flank fumaroles are compared with other sources.

tal crust is subducted to a depth of, at most, ~40 km below the Savu Basin north of western Timor, and so that only old (Jurassic) oceanic crust lies below Flores (and Pantar). The previous studies of arc helium^{1,2} show, however, that subduction of oceanic crust does not result in low (radiogenic) $^3\text{He}/^4\text{He}$ ratios, so that unless the Egon samples have been contaminated with near-surface radiogenic helium, a source of continental material is required at depth. Hamilton (personal communication) suggests that subducting sediments from abyssal fans north-west of Australia, derived from Precambrian crust, could well be present below eastern Flores and the transition zone to supply the observed helium. But McCaffrey *et al.*²⁶, who carried out a detailed microearthquake study in the Savu Basin, found a sharp seismic boundary between oceanic and continental crust striking ~N 65° E between Timor and Pantar, which they interpreted as a bend in the subducting slab of continental crust, extending to a depth of 150 km. This would put continental material beneath Pantar and probably beneath Solor (ref. 4, Fig. 78) at the eastern end of Flores. Our data are not sufficient for us to choose between these interpretations of the geophysical results but, with more extensive sampling across the ^3He transition zone, it may be possible to track the subducted continental crust across this zone.

Figure 2 also shows the striking antithetic correlation between helium and strontium isotopes along the Sunda arc. A similar correlation has been observed in restricted regions of the Mid-Atlantic Ridge²⁷, but this is the first clear case for two-component mixing of helium and strontium over a major segment of an active plate boundary. The antithetic He-Sr relationship is not present in the Banda arc: $^3\text{He}/^4\text{He}$ ratios are almost constant whereas $^{87}\text{Sr}/^{86}\text{Sr}$ ratios continue to increase along the arc. The lowest $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the Indonesian arcs is ~0.7040 in the lavas of Bali¹⁰; these define a 'Bali low' in Fig. 2, close to the value for the MORB-like endmember, and correspond to an increased $^3\text{He}/^4\text{He}$ ratio: a 'Bali high' relative to the rest of the arc. □

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Leakage of helium from the Pannonian basin

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THE aquifer system of the Great Hungarian Plain covers 50,000 km² and contains helium with an isotopic composition that indicates regional loss of both radiogenic-crustal and mantle-derived helium. Mantle-derived melts must be present at depth over much of the region. Here we show that the mantle helium flux through the basin may be supported by the addition of 20 m of melt per Myr. Considerations of hydrogeology and helium abundances show that the rate of loss of radiogenic helium from the basin may exceed the crustal helium production rate.

All continental ground waters contain small amounts of dissolved helium, often considerably in excess of the equilibrium saturation value. Most of this helium is generated by radioactivity and other nuclear processes within the crust. Such helium is characterized by its isotopic composition,

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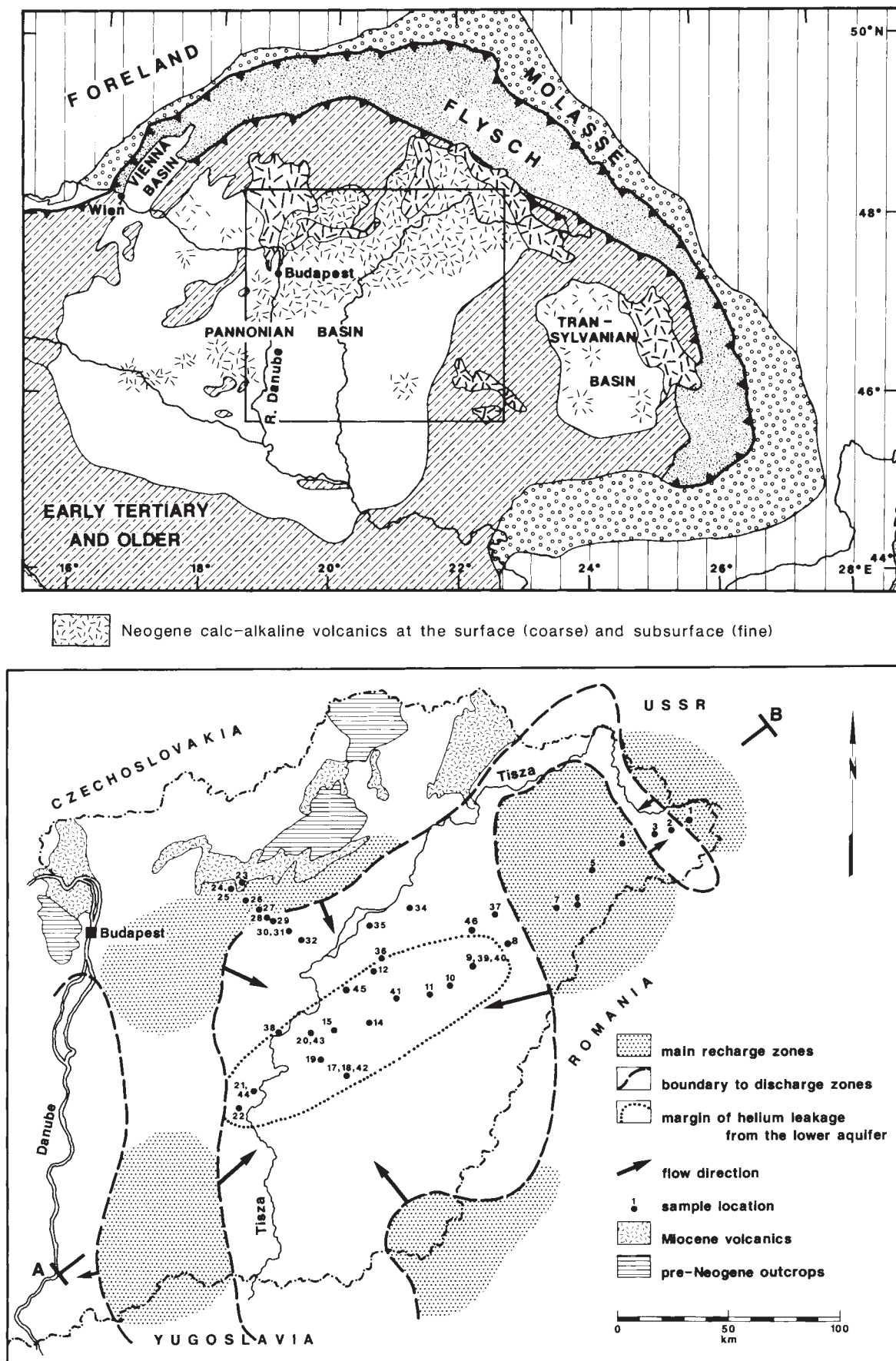


FIG. 1 Regional geology and hydrology of the Great Hungarian Plain. *a*, Tectonic sketch map of the intra-Carpathian basins, showing the location of the study area (shown in *b*). The Pannonian basin is filled with late Tertiary and Quaternary sediments. Toothed lines are major thrusts. *b*, The Great

Hungarian Plain showing the regional aquifer system groundwater flow directions and the localities at which it was sampled. Numbers refer to Table 1. A-B shows the line of the section depicted in Fig. 2.

$R = {}^3\text{He}/{}^4\text{He} \sim 10^{-8}$. In some areas, however, volatiles escape from the mantle or lower crust and accumulate in the ground-water system. The presence of another helium component derived from the mantle may be established by its distinctive isotopic composition ($R \approx 10^{-5}$).

In principle the fluxes of both mantle-derived helium and radiogenic crustal helium may be estimated if the characteristics of the aquifer are known. An ideal case would be a regional groundwater system of known area and well defined limits, for which the recharge and discharge were known and in balance. For such a result to be regionally significant, the extent of the aquifer system should be large by comparison with the thickness of the crust and direct losses of gas from the crust to the atmosphere should be small. In many respects the Great Hungarian Plain (GHP) within the Pannonian Basin meets these requirements, although there is no way of knowing how much gas is lost directly to the atmosphere and what proportion is trapped by the ground water.

The Pannonian basin in eastern Central Europe is an oval sedimentary basin, with a diameter of ~ 500 km (Fig. 1a). The sedimentary fill comprises early Miocene, Pliocene and Quaternary deposits that are predominantly clastic, often with significant amounts of rhyolite tuffs and lava flows¹. The basin is bounded by the Alps, Carpathians and Dinarides, and constitutes a closed hydrological system^{2,3}. The GHP forms the eastern part of the Pannonian basin and is a remarkably flat area between 80 and 200 m above sea level.

The Quaternary consists of poorly consolidated sand and clayey sand deposits (100–600 m) overlying 500–1,800 m of Pliocene lacustrine and fluvial alternating sands and clays. The underlying Miocene is a thick (up to 4,000 m) shale-rich sequence overlying a basement that varies in age from late Palaeozoic to Mesozoic and, locally, to Oligocene. The structural development of the GHP has been controlled by a set of trans-current faults striking east-north-east to west-south-west, and associated normal faults^{4,5}.

There are two water flow regimes of regional extent in the GHP^{2,6} (Fig. 2). The upper flow regime is essentially within the Quaternary. Meteoric water recharge occurs at topographic highs (Figs 1b and 2) and discharge occurs in topographic lows, particularly into the base of the Tisza River. A lower, dominantly connate, saline-water flow regime (Fig. 2) exists within the Pliocene and part of the Miocene. The Pannonian Basin is

artesian, with the upward flow controlled by overpressuring of deeper waters. Some communication between the two flow regimes occurs in the central part of the GHP (Fig. 2).

Hydrological observations indicate⁵⁻⁸ that the total ground-water flow in the GHP is $\sim 35 \text{ m}^3 \text{ s}^{-1}$. This value is based on some thousand pressure observations and permeability estimates from pumping experiments in boreholes. This is consistent with a previous estimate⁹ of $17 \pm 11 \text{ m}^3 \text{ s}^{-1}$ for the discharge of sub-surface water into the Tisza River system, which provides a lower limit for the discharge of the basin system as a whole. This latter estimate was derived from the progressive dilution of tritiated Tisza river water by non-tritiated sub-surface water over a distance of some 300 km.

The locations of samples analysed either in Cambridge or in Heidelberg are shown in Fig. 1b and the results summarized in Table 1. We collected the samples in conventional clamped annealed copper tubes and analysed them using very similar techniques. The methods used in Cambridge have been described previously¹⁰. We estimate associated experimental uncertainties to be $\leq 10\%$ for helium abundances and $\leq 3\%$ for isotope ratios.

Comparison of Table 1 with Fig. 1b shows that all samples contain more helium than the normal air saturation value ($4.64 \times 10^{-8} \text{ cm}^3 \text{ g}^{-1}$ at 10°C) and that the excess increases with distance from the recharge area and reaches a maximum two to three orders of magnitude higher than the saturation value. For some of the Pliocene waters we consider the measured value to be only a lower limit as their very low neon contents indicate that they have undergone degassing at some stage.

We obtain a crude estimate of the helium content of the discharging waters by averaging the values measured in Quaternary waters of the discharge zone. As far as we know, the Pliocene waters do not discharge directly to the surface but first mix with the overlying Quaternary waters.

The samples with the highest concentrations come from the central part of the discharge zone and may be the most representative of the helium contents of waters on their return to the surface. If so the simple average, $6.3 \times 10^{-6} \text{ cm}^3 \text{ g}^{-1}$, is a lower limiting value. This represents the helium that has been flushed from the rocks through which the waters have passed on their way from recharge to discharge. The R/R_A values where R_A is the ratio for the atmospheric standard given in Table 1 show that although most of the helium is radiogenic

FIG. 2 Generalized cross-section to show the water flow regimes in the sedimentary rocks of the Great Hungarian Plain. The section crosses in south-west/north-east direction the area of Fig. 1b. Key: 1, permeable and impermeable deposits at the surface; 2, highly permeable Quaternary beds; 3, Upper Pliocene consisting of impervious and permeable layers; 4, Pliocene sequence of permeable rocks containing fossil sea water and fresh water; 5, late and middle Miocene, mostly aquiclude; 6, late and middle Miocene volcanic rocks; 7, basement of the sedimentary basin consisting of Mesozoic or older rocks and their tectonic contact with Palaeogene flysch; 8, water flow direction in the upper aquifer (thin arrow) and lower aquifer (thick arrow).

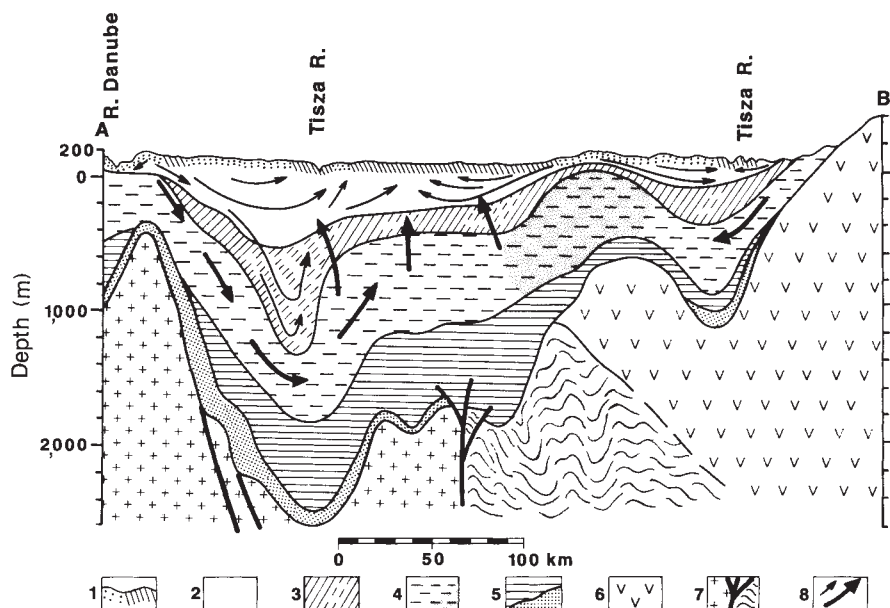


TABLE 1 Isotope data for Quaternary and Pliocene aquifers

Samples from Quaternary aquifers

a Recharge zone

Sample locality	Depth (metres)	Helium [†] (10 ⁻⁸ cm ³ g ⁻¹)	Neon [‡] (10 ⁻⁷ cm ³ g ⁻¹)	R/R _A [§]
1 Kőlcse*	90-116	11.1	—	—
2 Mánd*	73-120	32.6	—	—
3a Fehérgyarmat	82-96	25.6 (0.93)	—	0.33
3b Fehérgyarmat*	165-189	25.1	—	—
4 Mátészalka*	226-251	9.2	—	—
5a Nyírbátor	161-193	5.5	—	—
5b Nyírbátor*	297-313	11.4	—	—
6 Nyírlugos*	110-175	4.9	—	—
7 Nyíradony*	244-254	5.7	—	—
8a Debrecen	25-49	4.9	—	—
8b Debrecen	144-169	5.0	—	—
8c Debrecen	70-92	6.8	—	—
8d Debrecen*	138-181	21.3	—	—

b Discharge zone

9 Hajdúszoboszló*	<118	1,390 (117)	—	0.71
10 Kaba*	182-209	1,030	—	—
11 Püspökladány*	192-215	1,040	—	—
12 Kunmadaras	260-294	512 (34.7)	1.00	0.58
14b Kisujszállás	56-72	1,030	—	—
14c Kisujszállás	93-106	900	—	—
14d Kisujszállás	239-257	1,270 (126)	—	0.83
14e Kisujszállás	509-598	730	—	—
15 Örményes	258-286	408 (19.5)	—	0.42
17 Mezötúr	92-122	223 (21.1)	3.87	0.47
18 Mezötúr	161-170	309 (25.2)	1.45	0.69
19 Kétpo	390-405	782 (42.2)	1.82	0.47
20 Törökszentmiklós	239-281	208 (4.7)	2.59	0.22
21 Tiszakécske	211-220	182 (27.7)	1.27	1.25
22 Lakitelek	—	307 (52.8)	1.28	1.41
34a Tiszacsege	198-210	292 (9.9)	—	0.31
45 Kunhegyes	288-344	425	—	—
46 Balmazújvaros	85-98	286	—	—

Samples from Pliocene aquifers

a Recharge zone

23 Abasár*	11-19	5.3	—	—
24 Gyönyös*	140-194	7.2	—	—
25 Gyöngyös*	191-239	6.0	—	—
26 Karácsond*	274-330	193	—	—
27 Nagyfüged*	282-332	19.0	—	0.39
28 Tarnaméra*	435-594	98.6	—	—
29 Boconád*	446-467	46.6	—	0.35
30 Heves*	360-387	27.1	—	0.67
31 Heves*	674-772	169	—	0.23
32 Hevesvezekény*	541-568	40.6	—	—

b Discharge zone

34b Tiszacsege	1,085-1,271	484¶	0.44¶	0.32
35 Tiszafüred	719-970	229¶	0.91¶	0.11
36 Tiszaörs	716-1777	53.0¶	0.086¶	0.44
37 Hajdúböszörmény	598-636	8900	3.81	0.22
38 Szolnok	1,017-1,125	14.5¶	0.024¶	0.38
39 Hajdúszoboszló	381-434	277¶	0.17¶	0.72
40 Hajdúszoboszló	533-684	1,030	1.87	0.71
41 Karcag	1,190-1,334	128¶	0.06¶	0.25
42a Mezötúr	602-806	—	—	0.56
42b Mezötúr	924-1034	779	3.87	0.47
43 Törökszentmiklós	995-1127	1,510¶	0.28¶	0.83
44 Tiszakécske	925-1003	76	3.42	1.39
8e Debrecen	826-966	1,510	58.2	0.11

* Data from ref. 12.

† Total helium content (mantle-derived component in parentheses), assigned uncertainty $\pm 10\%$.‡ Total neon content, assigned uncertainty $\pm 20\%$.§ Measured $^3\text{He}/^4\text{He}$ ratio relative to an atmospheric standard.(R_A = 1.4×10^{-6}), corrected for atmospheric component using measured neon concentration following the method in ref. 21.

|| Values uncorrected for atmospheric component because of lack of neon concentration data.

¶ Low neon concentration indicates some helium depletion.

and probably of crustal origin a small but variable amount must in every case be derived from the mantle. Assuming a value of $R/R_A = 8.0$ for mantle helium the proportion varies from 1 to 16%.

We consider the origin and significance of these components separately. All ground waters from the Pannonian Basin analysed for helium isotopes so far^{11,12} (Table 1) have R/R_A values exceeding the radiogenic production ratio for crustal helium. This is also true for over one hundred other analyses we have made elsewhere in Hungary¹³. We consider the excess ^3He to be mantle derived and to be pervasively invading the crust, presumably along with other volatiles, over much of the Pannonian Basin. We obtain a total flow of $15 \text{ cm}^3 \text{ s}^{-1}$ (STP) for mantle-derived helium through the GHP from the product of the average mantle helium content of Quaternary discharge waters (Table 1) and the water flow rate ($35 \text{ m}^3 \text{ s}^{-1}$). Over the $5 \times 10^4 \text{ km}^2$ area of the GHP the $15 \text{ cm}^3 \text{ s}^{-1}$ mantle helium flow rate corresponds to $\sim 8 \times 10^4 \text{ atom } ^3\text{He m}^{-2} \text{ s}^{-1}$. This estimate is necessarily crude, because of the uncertainty in the average mantle helium content of the aquifer, but for the reasons given earlier it probably represents a lower limit and it does provide a useful comparison with the globally averaged mantle ^3He flux from the ocean ridges of $4 \times 10^4 \text{ atom m}^{-2} \text{ s}^{-1}$. This is several orders of magnitude higher than in stable, tectonic continental areas^{14,15}.

Mantle helium has been shown to escape through the crust over much of southern and western Europe^{14,16,17}. It was argued that the most plausible means of maintaining such a flux of mantle volatiles was by the emplacement of small volumes of mantle melt in the lower crust, but over a much wider area than the surface volcanic manifestations indicate. Surface Plio-Pleistocene basaltic volcanics are present only sporadically in Hungary such as in the Little Hungarian Plain to the west of the area studied here, but have also been recorded from a borehole in the Great Hungarian Plain¹⁸.

The amount of melt addition beneath the Pannonian Basin is uncertain and could be very small. Because helium behaves as a highly incompatible element and fractionates into the first few per cent of partial melt, the total amount of helium extracted by melting does not depend greatly on the size of the melt fraction. For example, at ocean ridges $\sim 4 \times 10^{-3} \text{ mol}$ of helium ($R/R_A \sim 8$) are released from the mantle for every cubic metre of basaltic ocean crust formed. If the mantle helium leaking from the Pannonian Basin is supported by basalt with a similar concentration of helium this would require¹⁴ the addition to the crust of $\sim 110 \text{ m}$ of basalt per million years. In a sub-continental environment, however, large melt fractions (for example, 10%) such as those at spreading ridges are unlikely and for a 2% melt fraction, which is more likely given the alkalic nature of Plio-Pleistocene basalts in the Pannonian Basin¹⁸, the rate of addition would be 20 m Myr^{-1} .

The greater part (>90%) of the helium contained in the aquifer system is radiogenic and has been derived from the underlying continental crust, where its generation is associated with the production of radiogenic heat with the approximate relationship $10^{12} \text{ atom } ^4\text{He J}^{-1}$ (ref. 19). The production of heat within the crust of the GHP is estimated to be 25 mW m^{-2} ($\pm 50\%$), which is within the range for average continental crust and corresponds to an equivalent flux of $2.5 \times 10^{10} \text{ atom } ^4\text{He m}^{-2} \text{ s}^{-1}$. We compare this with the value of $\sim 8 \times 10^{10} \text{ atom } ^4\text{He m}^{-2} \text{ s}^{-1}$ derived by dividing the total crustal helium flux by the area of the basin. Given the uncertainties associated with both values we do not regard them to be significantly different and, considering the persistence of relatively high sub-surface temperatures in Hungary²⁰ since the late Tertiary, it would be surprising if surface helium loss departed very greatly from the production

rate. If further work were to substantiate the difference, however, it would be necessary to consider possible addition of stored 'fossil' ^4He from the deeper aquifer systems.

We now consider the broader relationship between heat and helium in the Pannonian Basin, particularly in the light of the studies made on high-temperature hydrothermal vents on mid-ocean ridges¹⁵. No systematic relationship was found between water temperature and either isotopic composition or concentration of helium and it would perhaps be surprising if such a relationship existed. The aquifer system is known to have an important effect of surface heat flow in the Pannonian Basin^{2,20} but primarily by redistributing heat in the upper one or two kilometres of the crust, depressing temperatures in down-flow areas and elevating them where flows ascend. The major contributions to surface heat flow, including crustal radiogenic and sub-crustal sources, originate below the circulation depth. The circulations seem to be principally driven by hydrological head with only minor perturbations by convective thermal instabilities. By contrast, quite different factors control the helium inventory and its isotopic composition: in the case of the crustally derived radiogenic component, it is the age of the waters and to some extent variations in crustal chemistry; in the case of the mantle component, it is probably the distribution of faults and joints that localize the ascent of volatiles from below.

The rates of magmatic addition to the crust discussed earlier are sufficiently low that if they were more or less uniform across the basin, they would make an addition to the crustal heat flow that was undetectable against the major contributions from crustal radioactivity and conduction through the base of the lithosphere; together the latter sustain an average surface heat flow of $\sim 80 \text{ mW m}^{-2}$ in the Pannonian Basin¹⁹ whereas magmatic addition at a rate of 20 m Myr^{-1} would contribute only 2 mW m^{-2} . It also follows that the processes transporting helium released by the magma through the crust are unlikely to make a significant contribution to the transport of heat; although hard to substantiate, it is likely that the helium is transported by carbon-dioxide-rich fluids that are of insufficient mass to affect the temperature in the regions through which they pass. $\square$

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Boron isotope evidence for the involvement of non-marine evaporites in the origin of the Broken Hill ore deposits

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IDENTIFYING the palaeogeographic setting and mode of origin of stratabound ore deposits can be difficult in high-grade metamorphic terranes, where the effects of metamorphism may obscure the nature of the protoliths. Here we report boron isotope data for tourmalines from the early Proterozoic Broken Hill block, in Australia, which hosts giant lead–zinc–silver sulphide deposits. With one exception the $^{11}\text{B}/^{10}\text{B}$ ratios are lower than those for all other tourmalines from massive sulphide deposits and tourmalinites elsewhere in the world. We propose that these low ratios reflect leaching of boron from non-marine evaporitic borates by convecting hydrothermal fluids associated with early Proterozoic continental rifting. A possible modern analogue is the Salton Sea geothermal field in California.

The Broken Hill block is an inlier of early Proterozoic metamorphic and igneous rocks in western New South Wales, Australia (Fig. 1). Proterozoic rocks there consist of meta-sedimentary schists and gneisses and minor metavolcanic rocks^{1–3} that have undergone complex post-depositional deformation and regional metamorphism^{4–6}, including granulite-facies pressure and temperature conditions developed in the southern part of the block⁵. The palaeotectonic setting of the region is generally considered to represent an intracontinental rift^{2,3}.

Detailed mapping studies^{1–3} have recognized a coherent stratigraphy in the Broken Hill block (Fig. 2). The lowest stratigraphic units (Thackaringa Group and below) have recently been re-interpreted^{3,7} as fluvio-deltaic and lacustrine (or coastal sabkha) sequences, overlain by shallow marine sands and shales comprising the Broken Hill, Sundown and Paragon Groups; tholeiitic basalt flows, tuffs or sills (presently amphibolites) and possible rhyodacitic volcanics (now quartzo-feldspathic gneisses) are mainly within the Thackaringa and Broken Hill Groups. A variety of stratabound and stratiform mineralization occurs in these same two groups^{8,9} including the giant lead–zinc–silver lodes^{10,11} stratigraphically within the upper part of the Broken Hill Group.

The borosilicate tourmaline is an accessory gangue mineral in many massive sulphide deposits, particularly those in dominantly clastic metasedimentary terranes^{12,13}. Tourmaline is also the diagnostic mineral in stratabound tourmaline-rich rocks or tourmalinites, which are associated with a variety of stratabound (and commonly stratiform) mineral deposits^{12,14,15}. Like massive sulphide deposits, most tourmalinites are believed to form by syngenetic or diagenetic processes before regional metamorphism^{12–15}. Palmer and Slack¹⁶ recently determined the boron isotopic composition of tourmaline in over 40 stratabound massive sulphide deposits and tourmalinites from diverse geological and tectonic settings worldwide. Figure 3 shows the range of $\delta^{11}\text{B}$ values obtained for tourmaline separates from these

stratabound deposits ($n=52$) and for Broken Hill ($n=34$), together with the ranges observed for various fluid and solid boron reservoirs (the analytical details are given in ref. 16). The major control over the $\delta^{11}\text{B}$ values of the tourmalines is the composition of the boron source, especially the nature of foot-wall lithologies; secondary controls are water/rock ratios (indicated by $\delta^{18}\text{O}$ – $\delta^{11}\text{B}$ relationships) and seawater entrainment (indicated by local geological settings)¹⁶. Tourmalines from dominantly clastic metasedimentary and metavolcanic terranes have intermediate $\delta^{11}\text{B}$ values (–15.7 to –1.5‰), whereas tourmalines from marine metaevaporite and carbonate-associated terranes have higher $\delta^{11}\text{B}$ values (typically >0‰). In both groups, tourmaline $\delta^{11}\text{B}$ values are systematically lower than that of the fluid from which they precipitated and that of the solid reservoirs from which the boron was originally derived. Boron isotope systematics are such that the light isotope, ^{10}B , is preferentially partitioned into the solid phase during fluid–solid interactions^{16,17}. Under the pressure and temperature conditions inferred for massive sulphide and tourmalinite formation, tourmalines are interpreted to be 5–12‰ lighter than the boron-bearing fluid phase, mainly as a function of temperature–

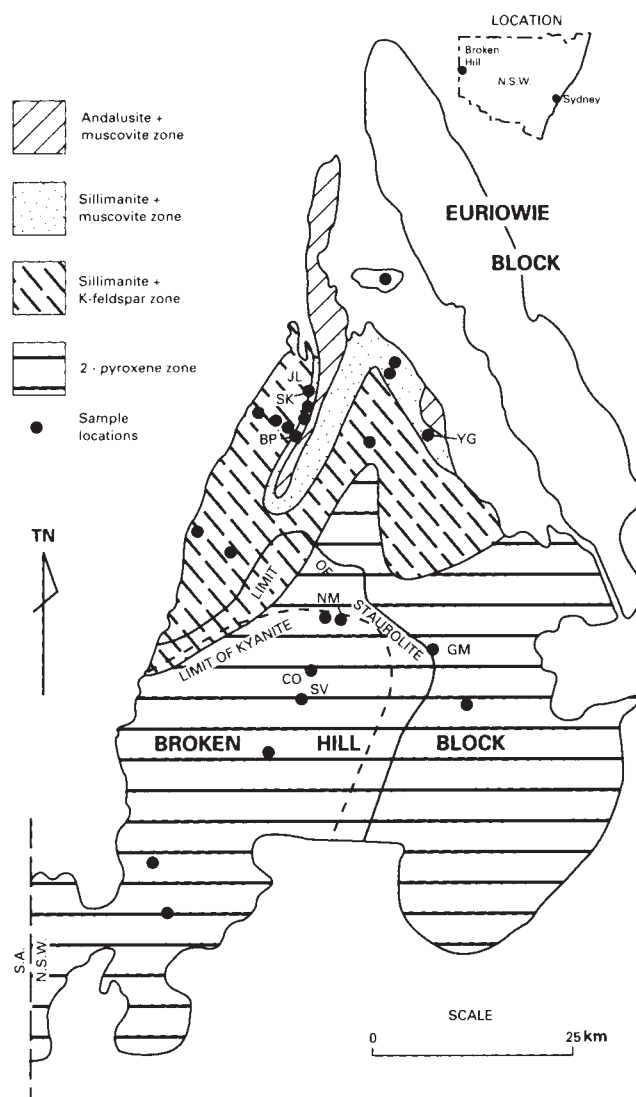


FIG. 1 Outline of the Broken Hill block showing metamorphic zones and sample locations. Prograde zones after Phillips⁵; retrograde zones (kyanite, staurolite) after Stevens⁶. We collected multiple samples at some localities. BP, Black Prince mine; CO, Corruga prospects; GM, Globe mine; JL, Jersey Lily mine; NM, Nine Mile mine; SK, Silver King West mine; SV, Stirling Vale homestead; YG, Yanco Glen area.

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dependent fractionation (ref. 16; M.R.P. and D. London, unpublished results). Little or no isotope fractionation takes place during hydrothermal leaching of boron from solid reservoirs^{18,19}.

Tourmaline-rich rocks are present in the Thackaringa, Broken Hill and Sundown Groups, but are most abundant in the upper part of the Broken Hill Group^{20,21}. Concentrations of tourmaline are known in pelitic and psammitic metasedimentary rocks, in stratabound and stratiform tourmalinites, in lead-zinc-silver lodes, in stratabound tungsten deposits, in metamorphic segregations and veins, and in granitic pegmatites. Tourmaline separates from these diverse occurrences in the Broken Hill block all have very low $\delta^{11}\text{B}$ values of -23.1 to -17.2% (Fig. 3). These values are unique relative to tourmalines from all other massive sulphide deposits and tourmalinites analysed to date¹⁶, except for one tourmalinite from the Archaean Isua Group in West Greenland ($\delta^{11}\text{B} = -22\%$, ref. 22).

We propose that the low $\delta^{11}\text{B}$ values for the Broken Hill tourmalines reflect derivation of boron from source(s) containing isotopically light boron. The only natural reservoirs (Fig. 3) known to have sufficiently low $\delta^{11}\text{B}$ values are borate minerals within non-marine evaporite sequences²³. Geological data from the Broken Hill block are consistent with this interpretation, including the occurrence of stratiform albitites²⁴ in the Himalaya Formation (Fig. 2) which are considered to be the metamorphosed equivalents of analcime-rich tuffs or sediments altered in an alkaline sodic environment^{3,25} and the presence of the Ettlewood Calc-Silicate Member at the base of the Broken Hill Group which is interpreted as a metamorphosed dolomite-chert unit^{3,26}. Further evidence for evaporites includes the occurrence of scapolite locally in the Redan Gneiss and Ettlewood Calc-Silicate Member³. Abundant magnetite disseminated in quartzofeldspathic rocks of the Thackaringa Group²⁴ suggests protoliths of oxidized red beds; local magnetite-sillimanite rocks imply the former existence of bauxites or laterites³. Also, N. Herriman (personal communication) has recognized apparent crystal casts of the evaporite minerals kainite and gypsum in relatively undeformed rocks from the Black Prince mine (Fig. 1), which locally contain well preserved sedimentary structures^{14,27}; the crystal casts may represent relict diagenetic minerals formed from a hypersaline interstitial brine.

Alternative interpretations of the Broken Hill tourmaline data include: (1) derivation of boron from low- $\delta^{11}\text{B}$ Proterozoic sea water; (2) increased isotope fractionation during low-temperature deposition or diagenesis; (3) metamorphic fractionation. The first possibility is unlikely because tourmalines from other massive sulphide deposits of similar ($1,750 \pm 50$ Myr) age,

such as those in the Baltic Shield of Finland and Sweden, have much higher $\delta^{11}\text{B}$ values¹⁶ (-12.3 to -2.0%). Because hydrothermal fluids readily leach boron from sediments, and because most sediments have high concentrations of boron relative to sea water, the $\delta^{11}\text{B}$ value of the fluid—and thus of precipitated tourmalines—is dominated by the $\delta^{11}\text{B}$ value of the sediment.

Low-temperature ($<200^\circ\text{C}$) formation of tourmaline could produce greater isotope fractionation between fluids and tourmaline and yield low $\delta^{11}\text{B}$ values¹⁶. This possibility cannot be completely excluded for Broken Hill, but there are several arguments against it. First, other stratabound lead-zinc deposits presumed to have formed at low temperatures such as those at Montauban (Quebec) and Balmat (New York) have associated tourmalines with much higher $\delta^{11}\text{B}$ values of $+1.6$ and $+6.7\%$, respectively; tourmaline from the geologically similar Broken Hill lead-zinc-silver deposit in the Cape Province of South Africa is also isotopically heavier¹⁶ (-13.8%). Second, no relationship exists between the $\delta^{11}\text{B}$ values of the Broken Hill tourmalines and their mineralogical, lithological or stratigraphic settings that might reflect different formation temperatures. Tourmalinites in the Globe mine section intimately associated with stratiform lead-zinc-silver lodes and manganese-garnet quartzites contain tourmaline with nearly identical $\delta^{11}\text{B}$ values (-22.5 to -20.9%) to those from tourmalinites associated with amphibolite and stratabound scheelite mineralization in the Corrua and Yanco Glen areas (-22.8 to -21.9%). These data from the Broken Hill Group are similar to those for the Thackaringa Group, such as at Stirling Vale where tourmalinites occur locally in bedded albitites ($\delta^{11}\text{B} = -19.7\%$). The similarity in $\delta^{11}\text{B}$ values of tourmalines from these very different environments (and in others throughout the Broken Hill block) is evidence for a common source of the boron or a common process by which they acquired their distinctive $\delta^{11}\text{B}$ values.

Metamorphic recrystallization of tourmaline could produce low $\delta^{11}\text{B}$ values by preferential fractionation of the heavy boron isotope, ^{11}B , into a fluid phase or into other aluminosilicate minerals. There is, however, no correlation between tourmaline $\delta^{11}\text{B}$ values and metamorphic grade in the region or with variations in mineral textures or mineral assemblages. Data for coarse-grained (>1 cm), metamorphically recrystallized tourmalines from the Silver King West mine ($\delta^{11}\text{B} = -21.3\%$) and the Jersey Lily mine (-17.2%) span a similar range of isotopic composition to that determined for fine-grained (<1 mm) tourmalines which apparently are not recrystallized. The relatively high value for the Jersey Lily sample may represent uptake of isotopically heavy boron released from the recrystalliz-

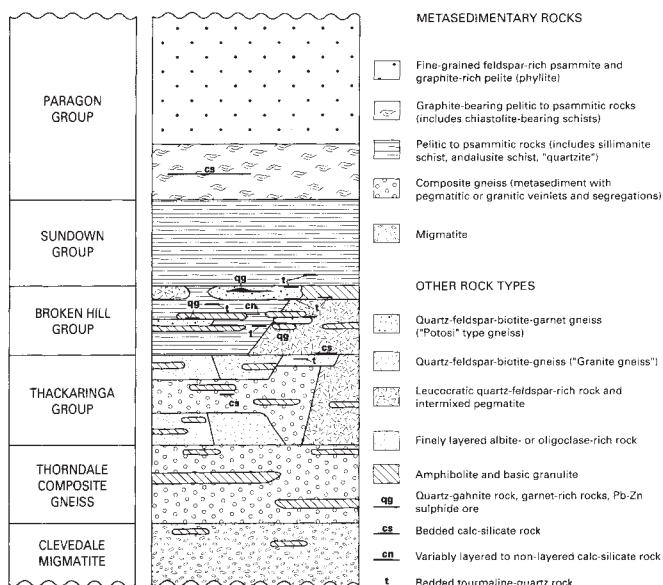
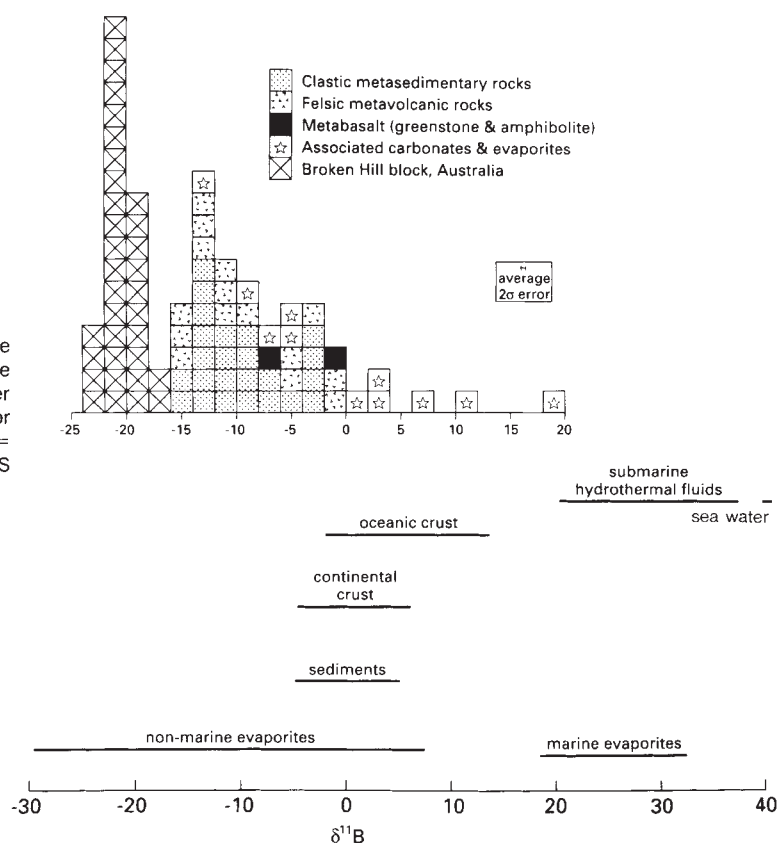


FIG. 2 Generalized lithostratigraphic sequence of the Broken Hill block after Willis *et al.*² and Barnes⁹. Total thickness of the sequence is $\sim 7-9$ km (refs 2, 3). Stratigraphic locations of important tourmalinites are schematically shown ('t'), based on work of Barnes^{8,9,27}, Willis *et al.*², and Stevens and Slack (unpublished results). Minor occurrences of tourmaline-rich veins, metamorphic segregations and pegmatites are not shown. The Himalaya Formation comprises upper part of the Thackaringa Group.

FIG. 3 Boron isotopic compositions of tourmaline (above) from the Broken Hill block compared with those from massive sulphide deposits and tourmalinites in geological settings elsewhere¹⁶. Lower scale shows the range of boron isotopic compositions identified for various fluid and solid boron reservoirs in nature^{18,19,23}. $\delta^{11}\text{B} = [({}^{11}\text{B}/{}^{10}\text{B})_{\text{sample}}/({}^{11}\text{B}/{}^{10}\text{B})_{\text{standard}}] - 1$, where the standard is NBS boric acid SRM 951.



ation of local boron-bearing silicates (such as sillimanite²⁸), but it clearly does not indicate significant boron loss during prograde metamorphism. The retrograde metamorphism^{6,29} common in parts of the Broken Hill block (Fig. 1) also has not substantially affected the boron isotopic composition of the tourmalines, as indicated by $\delta^{11}\text{B}$ values of -21.4% for metamorphically corroded tourmaline from a retrograde shear zone near the Nine Mile mine, and -20.1% for unaltered tourmaline from the same area but outside the shear zone. The -1.3% shift in $\delta^{11}\text{B}$ for this corroded tourmaline is in agreement with predicted isotope behaviour at high temperatures (M.R.P. and D. London, unpublished results), but is not significant relative to the range of $\delta^{11}\text{B}$ values measured for Broken Hill. We conclude that the mechanical and chemical stability of tourmaline, together with the lack of other boron-rich mineral hosts in the district, prevented major boron isotope fractionation during metamorphism in the Broken Hill block. This interpretation is consistent with tourmaline $\delta^{11}\text{B}$ data from greenschist-, amphibolite-, and granulite-facies terranes elsewhere¹⁶.

Our model, based on boron isotope and other data, suggests that parts of the Thackaringa Group originally contained non-marine evaporites. Borates within such evaporites provide a source of abundant, readily extractable boron; concentrations of saline minerals and carbonates are also likely to have been present in such a setting. Deposition of the overlying Broken Hill Group in a shallow marine basin^{3,7} was apparently associated with continental rifting and the development of a convecting hydrothermal system that extended into the underlying sediments of the Thackaringa Group, leaching them of boron, salts and, perhaps, sulphur and metals. The major stratabound lead-zinc-silver ores were then deposited by exhalative and/or diagenetic replacement processes together with siliceous lode rocks and manganese-iron sediments (such as garnet quartzites); coeval tourmalinites and stratabound tungsten mineralization formed generally in distal sedimentary environments. Widespread tourmalinites in the Broken Hill block, and the abundance of carbonate gangue in the main lead-zinc-silver lodes,

may be explained by a source reservoir of non-marine evaporitic borates and carbonates in the Thackaringa Group. We suggest that the restriction of abundant carbonate to the lode horizons⁹⁻¹¹ in the upper Broken Hill Group reflects boiling of carbon-dioxide-rich hydrothermal fluids in a shallow marine basin, in which the carbon dioxide was derived by leaching of carbonate-bearing evaporites in the underlying Thackaringa Group. Evaporite salts in the Thackaringa Group leached by convecting hydrothermal fluids and resultant increased metal-chloride complexing may have been critically important for transporting the huge quantities of lead, zinc and silver deposited in the main lodes.

A possible modern analogue of the Broken Hill block is the Salton Sea geothermal field in southern California where deep geothermal fluids that interact with Pliocene lacustrine and non-marine evaporite sequences^{30,31} have high concentrations of sodium, calcium, potassium, iron, manganese, zinc, lead, chlorine, lithium and boron³². This geothermal system is actively depositing iron-zinc-lead-copper sulphide minerals with $\delta^{34}\text{S}$ values from -9 to $+9\%$ (similar to sulphides of the Broken Hill lode^{33,34}) which reflect sulphur derivation by the hydrothermal reduction of anhydrite-derived sulphate in evaporitic sediments at depth³⁵. Continued rifting of the Salton Trough and transgression by sea water from the Gulf of California could produce a geological setting similar to that postulated for Broken Hill and an environment favourable for the formation of stratabound lead-zinc-silver mineralization and related tourmalinites. □

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Wavelength sensitivity in blindsight

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BLINDSIGHT—the residual visual functions observed in visual-field defects resulting from destruction of part of the primary visual cortex (striate cortex) even though visual stimuli presented in the field defect are not consciously perceived—has generated new insights into the nature of consciousness and the role of the extrastriate pathways in visual processing. Some patients can detect and localize unseen stimuli when they are required to guess^{1–4}. Discrimination of movement^{5,6}, flicker^{5,7} and orientation^{4,8} may also be present, but residual colour discrimination is controversial. Negative results (see ref. 9 for review) imply that only the pathways from eye to striate cortex can transmit information about colour in primates. By measuring sensitivity to light of different wavelengths in patients with blindsight we show that spectral sensitivity in the blind fields is surprisingly high, with a reduction of only 1 log unit or less across the visible spectrum. It is also essentially normal in form, whether the patients are adapted to light or dark. The shift in peak sensitivity from medium to shorter wavelengths in adaptation to the dark (the Purkinje shift) and the presence of discontinuities in the light-adapted curve together show that blindsight involves both rod and cone contributions, and that some colour opponency remains. As colour opponency requires input from primate beta retinal ganglion cells, two-thirds of which degenerate transneurally after a striate cortical lesion in juvenile monkeys¹⁰, our results show that the surviving subpopulation of primate beta cells is functional.

The investigation of blindsight provides the only available behavioural method of isolating and studying the properties of the extra-geniculostriate cortical visual pathways in man, and can show which aspects of visual coding require the striate cortex and which do not. Damage to the striate cortex eventually leads to the death of the majority of ganglion cells in the corresponding part of the retina in man¹¹, and also in monkeys, where the degeneration is selective for, and involves up to 80% of, the colour opponent primate beta ($P\beta$) cells¹⁰. To see whether

the pathways from the degenerated hemiretina can process wavelength information we measured spectral sensitivity in the normal and blind fields of three patients in whom blindsight had been previously demonstrated⁹, and in two normal observers. We used both adaptation to the light and dark because the contradictory reports on colour discrimination in blindsight^{4,5,7,9,12–16} include a demonstration that spectral sensitivity of totally destriated monkeys is characteristic of rod receptors regardless of adaptation level¹⁵, indicating that only the colour-blind rod channel is effective.

The subjects were tested in a Tübinger perimeter, using monocular viewing such that their field defect was on the temporal side, corresponding to the normally more sensitive nasal retina. The field defects of the three patients are shown in Fig. 1. Stimuli were presented at positions 10° eccentric from the fixation axis, permitting a comparison of sensitivity at mirror-symmetric positions in the hemifields. While held by a head and chin rest, the subject fixated a central dim red point. The viewing eye was monitored through an infrared video system. Nine coloured targets (116', 200 ms) from 450 to 660 nm were used. Each was produced by a narrowband, 9–13 nm, interference filter. In the normal visual hemifield detection thresholds were repeatedly measured by progressively increasing stimulus intensity by 0.05 log units until the subject reported seeing the target on three consecutive presentations. For the blind field a guessing paradigm was used. Blank trials (on which no target

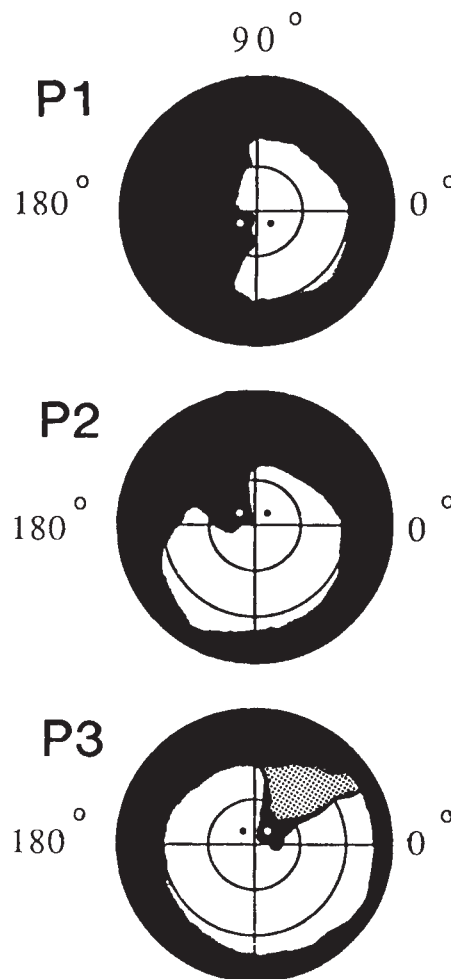
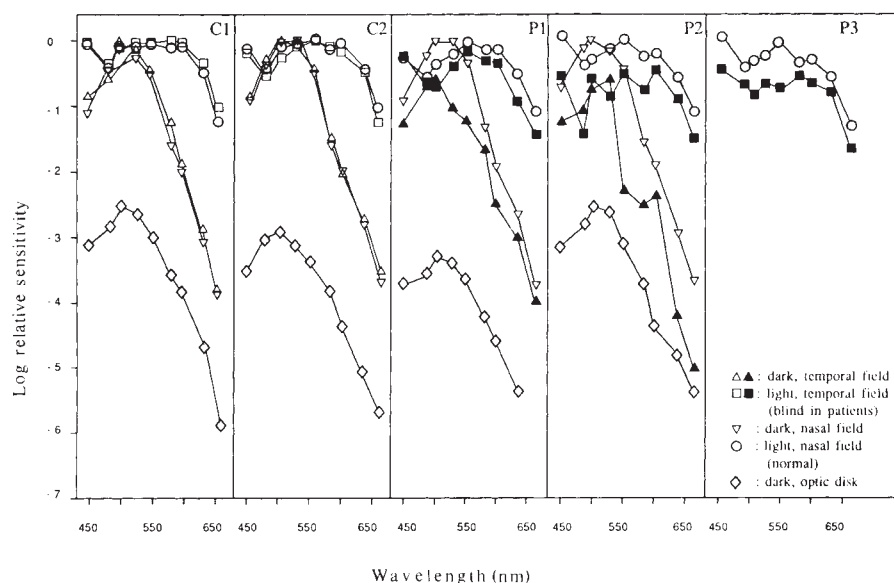


FIG. 1 Visual fields of patients plotted by moving a 320 cdm^{-2} 44' white target on a 10 cdm^{-2} background from the periphery towards the fixation point. The stippled area in P3 was amblyopic, as revealed by stationary flashed targets. Black or white spots at 10° eccentricity indicate the position where coloured targets were presented in intact or blind fields respectively.

FIG. 2 Relative spectral sensitivity at an eccentricity of 10° in two normal subjects and in the patients whose field defects are shown in Fig. 1. In each graph the most sensitive dark-adapted value is scaled to zero. The light-adapted values have been raised by 4.3(C1), 3.9(C2) and 3.7(P1,P2) log units so that the envelopes of the light- and dark-adapted curves can be readily compared. The key to the symbols is below the curves for P3, who was not tested when dark adapted. Black symbols refer to blind fields.



was presented) and stimulus trials, with an overall ratio of 4:1, were presented randomly, and the patient was asked to guess whether a light had been presented. After each batch of up to 250 trials, target luminance was increased by 0.1 log units, until detection was statistically significant at the 5% level. If a different value was obtained when the measurement was repeated, the mean was used. Stimuli were presented on a white $3,500 \pm 1,000$ K background of low photopic (32 cdm^{-2}) or scotopic luminance (0.0001 cdm^{-2}) after 20 min of adaptation to the dark. Under scotopic conditions, straylight detection thresholds were determined by presenting the targets in the natural blind spots; under photopic conditions the targets could not be made intense enough to be detectable in the blind spot.

Figure 2 shows the spectral sensitivity curves. Note that sensitivity in the normal and blind fields has a single peak at 500 nm under adaptation to the dark, the smooth curve and steep decline above 550 nm being characteristic of rod vision. Sensitivity in the blind fields is normal, apart from being reduced by up to a log unit, and the curves are less smooth. Comparison with the straylight detection thresholds measured under adaptation to the dark for targets on the reflective natural blind spot, which are 2–3 log units higher than for nearby targets in normal retina, shows that as sensitivity in the blind field is reduced by not more than 1 log unit it is not based on the detection of light scattered onto the functionally normal retina. Under adaptation to the light there is no steep decline in sensitivity above 550 nm such as occurs under scotopic conditions; instead, the curve shifts towards the longer wavelengths, showing that cones are involved.

Whereas the sensitivity of the colour-opponent system, especially when tested with targets on a high photopic white surround, has peaks at about 450, 525–550, and 600 nm, and notches at 480 and 580 nm (refs 17–19) (their precise positions depending on intensity and colour temperature of the background) the luminance system has a single broad peak around 555 nm (ref. 19). Given that the more sensitive system will determine threshold, we assume some contribution of the luminance system in governing sensitivity around 550 nm that is in accordance with the lack of a clear notch at 580 nm in several photopic curves. The sensitivity of the luminance system, however, decreases smoothly towards the short wavelengths¹⁹, probably because the broadband channels receive little if any input from short wavelength ('blue') cones^{20,21}, and can therefore not be responsible for the prominent secondary increase in sensitivity from 480/500 nm to 450 nm. The presence of this increase in all photopic curves indicates that colour opponent

processes contribute to or even determine thresholds for the 450 nm blue stimulus. This is supported by the results of colour naming at threshold in the good field, which was faultless for the blue and red, but uncertain and occasionally wrong for green and yellow stimuli.

Together, our results show that spectral sensitivity as measured under the present conditions differs between normal and cortically blind hemifields only in terms of absolute sensitivity. This difference is fairly small and is not an artefact of comparing different detection methods because it was not altered when the same guessing paradigm was applied in the normal field. There is no evidence that sensitivity is mediated solely by rod receptors, as this would abolish the marked difference between curves measured under adaptation to the light and dark. All measurements made under photopic conditions show contributions from cone receptors. The notch or shoulder at 580 nm and the rise in sensitivity from 480 to 450 nm provide evidence for colour opponent processes. If chromatic opponency does remain in blindsight, genuine wavelength discrimination may also be possible. This can now be tested by presenting coloured stimuli that are matched for luminous efficiency on the basis of the patient's own spectral sensitivity. Thus far, evidence indicates that the subpopulation of surviving P β ganglion cells, which are the only known ganglion cells to provide colour-opponent signals, is still functional. As chromatic opponency has never been detected in the midbrain visual pathway (a favourite candidate for the mediation of blindsight) remaining retinal projections to the thalamus and from there to extrastriate cortex may be involved^{22,23}. □

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Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells

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ACTIVATION of NMDA (*N*-methyl-D-aspartate) receptors by neurotransmitter glutamate stimulates phospholipase A₂ to release arachidonic acid^{1,2}. This second messenger facilitates long-term potentiation of glutamatergic synapses in the hippocampus³, possibly by blocking glutamate uptake^{4,5}. We have studied the effect of arachidonic acid on glutamate uptake into glial cells using the whole-cell patch-clamp technique to monitor the uptake electrically^{6,7}. Micromolar levels of arachidonic acid inhibit glutamate uptake, mainly by reducing the maximum uptake rate with only small effects on the affinity for external glutamate and sodium. On removal of arachidonic acid a rapid (5 minutes) phase of partial recovery is followed by a maintained suppression of uptake lasting at least 20 minutes. Surprisingly, the action of arachidonic acid is unaffected by cyclo-oxygenase or lipoxygenase inhibitors suggesting that it inhibits uptake directly, possibly by increasing membrane fluidity. As blockade of phospholipase A₂ prevents the induction of long-term potentiation (LTP)⁸, inhibition of glutamate uptake by arachidonic acid may contribute to the increase of synaptic gain that occurs in LTP. During anoxia, release of arachidonic acid⁹ could severely compromise glutamate uptake and thus contribute to neuronal death.

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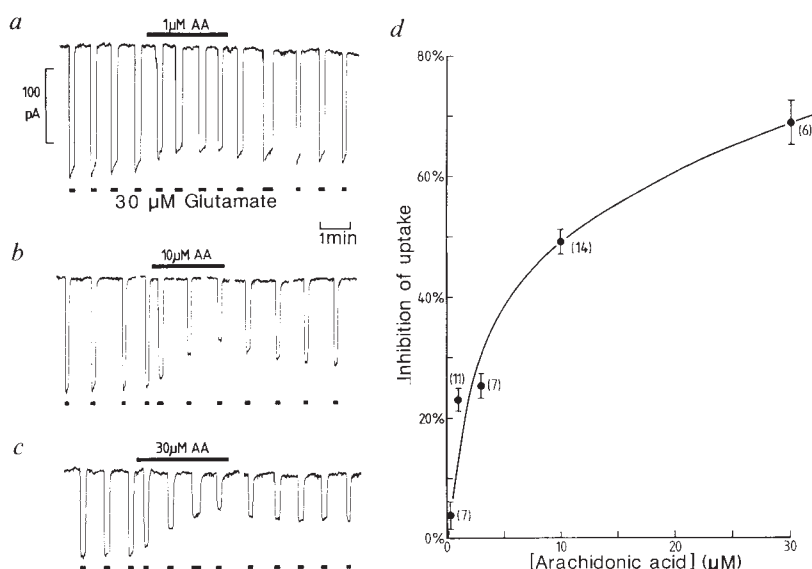
Application of L-glutamate to glial (Müller) cells from the salamander retina evokes an inward current reflecting glutamate uptake by an electrogenic carrier that transports Na⁺ into the cell^{6,7}. A response with similar characteristics has recently been demonstrated in mammalian glial cells^{10,11}; we used salamander glia for this study because of the large uptake current they exhibit. Glutamate uptake was monitored as the inward current evoked by 30 μ M glutamate in isolated glial cells voltage-clamped to -40 mV. Arachidonic acid produced a slowly developing inhibition of glutamate uptake (Fig. 1a–c). The uptake current was halved by 2 minutes' exposure to 10 μ M arachidonic acid, and significant inhibition was seen with 1 μ M arachidonic acid (Fig. 1d).

The dependence of the uptake current on external glutamate and external sodium concentrations, measured before and after applying arachidonic acid, showed that arachidonic acid suppresses uptake largely by reducing the maximum uptake rate, with a small increase in the apparent affinity for glutamate and a small decrease in the apparent affinity for external sodium (Fig. 2a, b).

The mechanism of action of arachidonic acid was investigated pharmacologically. Ouabain (10⁻⁴ M) did not prevent the inhibition produced by arachidonic acid (Fig. 3a), which was therefore not an indirect result of intracellular Na⁺ accumulation following inhibition of the sodium pump by arachidonic acid⁴. The cyclo-oxygenase inhibitor indomethacin (5 μ M) had no effect on the uptake inhibition (Fig. 3b), ruling out the possibility that it depends on the conversion of arachidonic acid to prostaglandin derivatives. Nordihydroguaiaretic acid (NDGA, 10 μ M), a lipoxygenase inhibitor, produced a slowly developing inhibition of uptake similar to that seen with arachidonic acid (possibly by inhibiting breakdown of arachidonic acid produced by the glial cell itself), but did not prevent inhibition of uptake by arachidonic acid (Fig. 3c). Metabolites of arachidonic acid like HPETE¹² are therefore not responsible for the inhibition of

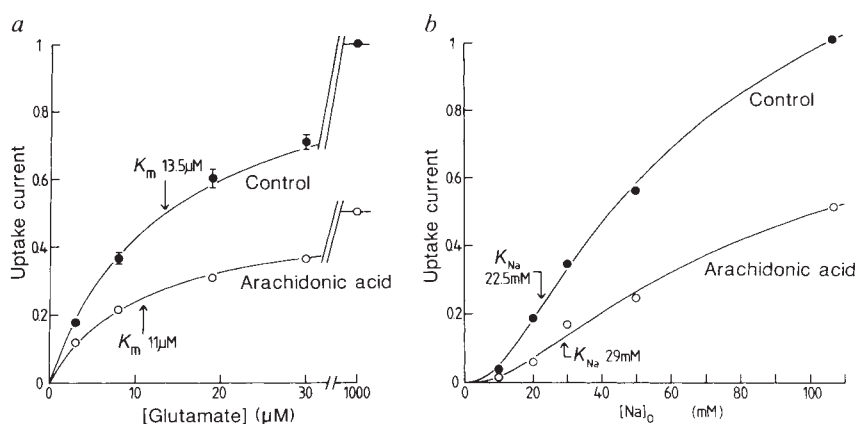
FIG. 1 Arachidonic acid inhibits glial cell glutamate uptake. *a*, *b* and *c*, current changes in three different Müller cells voltage-clamped to -40 mV during repeated application of glutamate (30 μ M) (timing shown by black bars below each record). Glutamate evokes an inward current (downward deflection) reflecting electrogenic glutamate uptake⁶. Application of 1 μ M (*a*), 10 μ M (*b*) or 30 μ M (*c*) arachidonic acid produces a slowly developing inhibition of the glutamate-evoked current. *d*, Percentage suppression of glutamate uptake (mean $\pm$ s.e.) as a function of arachidonic acid concentration measured after 2 min in the presence of arachidonic acid (number of cells shown by each point; smooth curve drawn by eye). During prolonged recordings even in the absence of arachidonic acid there is a slow decay of glutamate-evoked current, typically 2% per min, which correlates with a decrease in cell capacitance (measured from the current response to voltage jumps). To compensate for this loss of cell area, which would otherwise lead to an overestimate of the suppressive effect of arachidonic acid, glutamate-evoked currents were normalized by the cell capacitance measured before and in the presence of arachidonic acid. Normalized uptake current declined much more slowly over a prolonged recording period than did the un-normalized current (see Fig. 4).

METHODS. Glial (Müller) cells isolated from the tiger salamander retina⁶ were whole-cell clamped using pipettes containing (mM): KCl, 95; HEPES, 5; NaCl, 5; Na₂ATP, 5; MgCl₂, 7; CaCl₂, 1; K₂EGTA, 5; the pH was adjusted to 7.0 with 14 mM KOH. External solution contained (mM): NaCl, 105; KCl, 2.5;



CaCl₂, 3; MgCl₂, 0.5; glucose, 15; HEPES, 5; BaCl₂ (to block the cells' potassium conductance), 6. The pH was adjusted to 7.3 with 2 mM NaOH. Temperature, 20–23 °C. Arachidonic acid (Sigma) was stored under nitrogen at -20 °C as a 10 mM stock suspension in water or occasionally in DMSO, which gave similar results. When required this stock was thawed, sonicated for 5 min, and added to the superfusion solution, which was then sonicated for 5 min.

FIG. 2 Effect of arachidonic acid on the glutamate- and sodium-dependence of glutamate uptake. *a*, Dependence of uptake current (at -40 mV) on glutamate concentration before ($\bullet$) and after ($\circ$) 3 min in the presence of $10 \mu\text{M}$ arachidonic acid. All data normalized to the saturating response before arachidonic acid. Mean ($\pm$ s.e.) data from 10, 11, 5 and 6 cells for 3, 8, 19 and $30 \mu\text{M}$ glutamate. Curves through the points are Michaelis-Menten relations with $K_m=13.5 \mu\text{M}$, $V_{\text{max}}=1$ before, and $K_m=11 \mu\text{M}$, $V_{\text{max}}=0.5$ after arachidonic acid. Arachidonic acid halves the V_{max} of uptake with a small increase in apparent glutamate affinity. *b*, External sodium-dependence of the uptake current (at -40 mV) evoked by $30 \mu\text{M}$ glutamate before ($\bullet$) and after ($\circ$) 3 min in $10 \mu\text{M}$ arachidonic acid. Data normalized to control response in 107 mM $[\text{Na}]_o$. Mean data (s.e. < symbol size) from 3, 5, 11 and 5 cells in 10, 20, 30 and 50 mM $[\text{Na}]_o$. Smooth curves are empirical fits with the form: $V_{\text{high Na}} \{ [\text{Na}]_o / ([\text{Na}]_o + K_{\text{Na}}) \}^3$, with $K_{\text{Na}}=22.5 \text{ mM}$, $V_{\text{high Na}}=1.77$ before, and $K_{\text{Na}}=29 \text{ mM}$, $V_{\text{high Na}}=1.05$ after arachidonic acid. On this basis, most of the uptake inhibition produced by arachidonic acid in 107 mM $[\text{Na}]_o$ in Fig. 1 results from a decrease in



the rate of uptake that would be observed in saturating $[\text{Na}]_o$, although there is some contribution from the decrease in apparent affinity for Na_o . Fitting the data with the Hill equation instead of a power of the Michaelis-Menten relation led to the same conclusion.

glutamate uptake. Oleic acid inhibited uptake approximately half as well as arachidonic acid (Fig. 3*d*). Phorbol ester, which stimulates protein kinase C by 20–60%¹³, had little effect on uptake (Fig. 3*e*), ruling out the possibility that arachidonic and oleic acids reduce uptake by stimulating this kinase¹³. The longer *cis*-unsaturated fatty acid docosahexaenoic acid, which has no effect on protein kinase C¹³, produced an inhibition of uptake similar to that produced by arachidonic acid, and the protein kinase inhibitor H-7 had no effect on the action of arachidonic acid (see Fig. 3*e* legend).

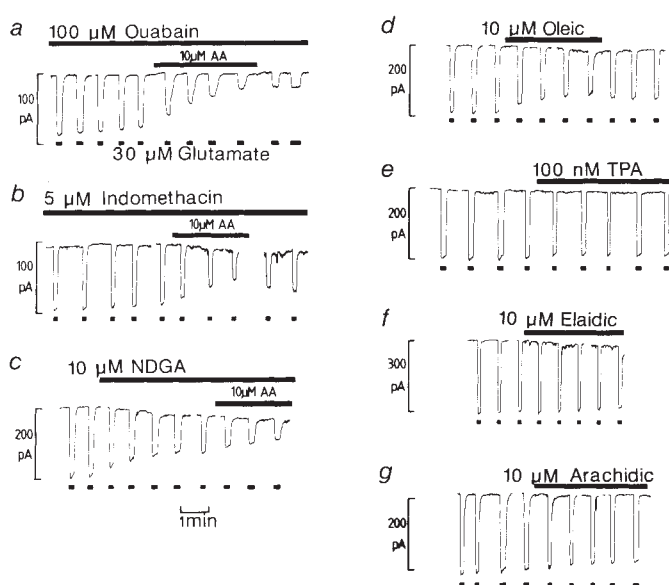
To test whether the effects of arachidonic and oleic acids are a consequence of the lipid disruption produced by the kinked *cis* double bonds in their structures (20:4 and 18:1 respectively), we applied two linear analogues of these molecules. Elaidic acid (18:1), a homologue of oleic acid with a *trans* instead of a *cis* double bond, produced only one quarter of the uptake inhibition

produced by oleic acid (Fig. 3*f*). Similarly arachidic acid (20:0), the saturated analogue of arachidonic acid, produced only about one fifth of the inhibition that arachidonic acid did (Fig. 3*g*).

These results are consistent with the inhibition of uptake produced by arachidonic acid being due to the disruption of membrane lipid organization (increase in membrane fluidity) caused by the introduction of unsaturated fatty acid into the membrane. The messenger actions of arachidonic acid are usually attributed to its lipoxygenase and cyclo-oxygenase derivatives. The direct action of arachidonic acid we propose here is similar to that suggested for the effect of arachidonic acid on the sodium pump¹⁴. Changes of membrane fluidity have also been proposed to regulate phosphate and glucose uptake in the kidney¹⁵.

The kinetics of action of arachidonic acid are of interest with respect to the possible role of arachidonic acid in long-term

FIG. 3 Pharmacology of the uptake inhibition. Uptake measured as the current (normalized by capacitance for quantitative analysis, see Fig. 1) evoked at -40 mV by $30 \mu\text{M}$ glutamate. *a*, Ouabain does not prevent the uptake inhibition produced by $10 \mu\text{M}$ arachidonic acid. Mean ($\pm$ s.e.) inhibition produced in three cells by 2 minutes' arachidonic acid in the presence of 10^{-4} M ouabain was $55.6 \pm 2.7\%$, similar to that seen without ouabain (Fig. 1). *In vivo*, where Na_i is not controlled by the patch pipette, accumulation of Na^+ inside the cell, following inhibition of the sodium pump⁴ by arachidonic acid, may contribute to inhibition of glutamate uptake. *b*, Indomethacin ($5 \mu\text{M}$, for 25 min) does not prevent the uptake inhibition produced by $10 \mu\text{M}$ arachidonic acid. Mean inhibition produced by 2 minutes' arachidonic acid after 20–30 min in indomethacin was $47.9 \pm 5.3\%$ ($n=3$), similar to that without indomethacin. In toad bladder, $6 \mu\text{M}$ indomethacin inhibits cyclo-oxygenase by $>90\%$ ²¹. *c*, Nordihydroguaiaretic acid (NDGA, $10 \mu\text{M}$) does not prevent the uptake inhibition produced by $10 \mu\text{M}$ arachidonic acid. NDGA itself reduced the uptake current (by $52.8 \pm 2.1\%$ after 8 min in NDGA, $n=9$), and subsequent application of arachidonic acid further reduced uptake by $45.1 \pm 4.7\%$ ($n=10$, after 2 min), similar to the inhibition seen without NDGA. Estimates of the 50% inhibitory concentration for NDGA block of lipoxygenase in rat and human are $\sim 0.9 \mu\text{M}$ and $\sim 3 \mu\text{M}$, respectively^{22,23}. *d*, Oleic acid, with one *cis* double bond in its structure, unlike the four in arachidonic acid, reduces uptake, but to a lesser degree than arachidonic acid. Mean reduction produced by 2 min of $10 \mu\text{M}$ oleic acid was $28.9 \pm 1.0\%$ (seven cells). *e*, Arachidonic and oleic acids do not act by stimulating protein kinase C. Phorbol 12-myristate 13-acetate (TPA, 100 nM), which activates protein kinase C by 20–60%¹³, has little effect on uptake (mean reduction in five cells after 4.5 min in TPA was $5.5 \pm 0.9\%$). Similar results were obtained with 10^{-6} M pipette $[\text{Ca}^{2+}]$, rather than 10^{-7} M $[\text{Ca}^{2+}]$ pipette as here. Furthermore, docosahexaenoic acid ($22:6$, $10 \mu\text{M}$ for 2 min), which has no effect on protein kinase C¹³, reduced the uptake current by $58.9 \pm 2.6\%$ ($n=5$, data not shown). Including the protein kinase inhibitor H7 ($340 \mu\text{M}$) in the patch pipette had no effect on the uptake inhibition produced by arachidonic acid in five cells. *f*, Elaidic acid has much less effect on



uptake than does its *cis* homologue oleic acid. Mean reduction of uptake after 2 min in $10 \mu\text{M}$ elaidic acid was $6.7 \pm 1.8\%$ (nine cells). *g*, Arachidic acid has much less effect on uptake than does its polyunsaturated analogue arachidonic acid. Mean reduction of uptake after 2 min in $10 \mu\text{M}$ arachidic acid was $10.4 \pm 1.3\%$ (eight cells). Drugs were made up as stock solutions in dimethylsulphoxide (DMSO) (0.1% final concentration; DMSO was also added to control solutions). Gaps in the records are where voltage pulses were applied to measure cell capacitance.

potentiation. We compared the time course of the uptake current in nine cells to which arachidonic acid was applied with the time course in 10 cells to which no arachidonic acid was applied (Fig. 4). The cells treated with arachidonic acid showed an inhibition of uptake as in Fig. 1 and, on removal of arachidonic acid, a fast (5 min) partial recovery followed by a prolonged period (>20 min) over which uptake remained significantly inhibited.

The results presented here are relevant to understanding the neuronal death produced by brain anoxia, and the synaptic gain increase that occurs during long-term potentiation. In brain ischaemia and anoxia the concentration of free arachidonic acid rises greatly⁹, and so glutamate uptake may be severely inhibited. The subsequent increase in the concentration of extracellular glutamate could contribute to neuronal death through the excessive activation of glutamate-gated ion channels¹⁶.

Induction of LTP leads to a release of arachidonic acid¹⁷, presumably by the activation of NMDA receptors^{1,2}; conversely, prevention of arachidonic acid release, by inhibition of phospholipase A₂, blocks the induction of LTP^{8,17}. Inhibition of glutamate uptake by arachidonic acid could increase the gain of synaptic transmission during LTP in two ways. First, a reduction of uptake could allow transmitter release to generate a higher glutamate concentration at the postsynaptic ion channels. Second, a reduction of uptake could increase the resting extracellular glutamate concentration and, because two or more glutamate molecules must bind to open a postsynaptic channel¹⁸, this could result in synaptically-released glutamate producing a larger postsynaptic response. Even a brief burst of arachidonic acid release, generated by the conditioning stimulus in LTP, could cause a prolonged blockade of glutamate uptake (Fig. 4), and hence a prolonged increase of synaptic gain. A decrease in

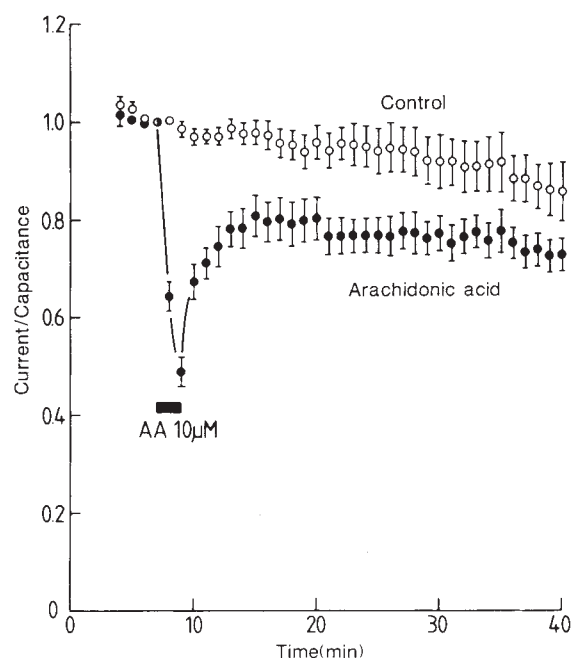


FIG. 4 Brief exposure to arachidonic acid produces a prolonged inhibition of glutamate uptake. Mean ($\pm$ s.e.) uptake current evoked by 30 μ M glutamate at -40 mV in 10 control cells ($\circ$) and 9 cells exposed to 10 μ M arachidonic acid for 2 min ($\bullet$). After arachidonic acid has approximately halved uptake, a rapid partial recovery is followed by a sustained period in which the cells treated with arachidonic acid show less uptake than the control cells ($P < 0.01$, 0.05 and 0.1 at 10, 20 and 30 min after removing arachidonic acid; 2-tailed t -test). Uptake current at each time was normalized by cell capacitance to compensate for a slow decrease in cell area with time, caused possibly by endocytosis or loss of cell blebs. Without capacity normalization, control cell currents decayed by 45% in 30 min; normalization reduced this to 12%. Prolonged inhibition of uptake by arachidonic acid was equally clear in the unnormalized data.

uptake may not have been detected¹⁹ during LTP because only a small fraction of uptake is suppressed (that in membranes near active synapses) and because uptake was assessed from the equilibrium level (not the initial rate) of glutamate accumulation.

The mechanism of LTP has been postulated to involve a messenger such as arachidonic acid increasing transmitter release from the presynaptic terminal. Our results indicate that an alternative possibility is a reduction of the removal of transmitter from the extracellular space. Such a mechanism could also explain the increased loss of glutamate into the extracellular fluid that occurs in LTP²⁰, which is usually attributed to increased glutamate release from presynaptic terminals. $\square$

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Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor

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CILIARY neurotrophic factor (CNTF) was originally characterized as a survival factor for chick ciliary neurons *in vitro*¹. More recently, it was shown to promote the survival of a variety of other neuronal cell types^{2,3} and to affect the differentiation of E7 chick sympathetic neurons by inhibiting their proliferation and by inducing the expression of vasoactive intestinal peptide immunoreactivity (VIP-IR)⁴. In cultures of dissociated sympathetic neurons from newborn rats, CNTF induces cholinergic differentiation as shown by increased levels of choline acetyltransferase (ChAT)⁵. This increase is paralleled by a reduction of tyrosine hydroxylase (TH) activity. Moreover, CNTF promotes the differentiation of bipotential O2A progenitor cells to type-2-astrocytes⁶ *in vitro*. To help establish which, if any, of these functions CNTF exerts *in vivo*, it is necessary to determine its primary structure, cellular

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expression, developmental regulation and localization. The complementary DNA-deduced amino-acid sequence and subsequent expression of cDNA clones covering the entire coding region in HeLa-cells indicate that CNTF is a cytosolic protein. This, together with its regional distribution and its developmental expression, show that CNTF is not a target-derived neurotrophic factor. CNTF thus seems to exhibit neurotrophic and differentiation properties only after becoming available either by cellular lesion or by an unknown release mechanism.

CNTF was purified from adult rat sciatic nerve as described previously⁵, using an additional HPLC purification step (for details see legend to Table 1). The amino-acid sequences of the various fragments determined by gas-phase microsequencing represented more than 50% of the protein. The peptide sequences thus obtained matched perfectly with those deduced from cDNA cloning as shown in Fig. 1.

To obtain RNA for molecular cloning, we used cultures of rat brain cells that previously had been shown to produce substantial quantities of CNTF (ref. 7). After various polymerase chain reaction steps, the nucleotide sequences of clones A, B, C revealed a short 5' untranslated region of 77 base pairs (bp) and an open reading frame of 600 bp, predicting a protein 200

amino acids long with a 3' untranslated region of 436 bp, which ends in a poly(A) tail (Fig. 1b). The initiation site for translation was localized at position 78–80 of the nucleotide sequence. A stop codon located 5' to this initiation site at position 72–74 and G residues in positions 75 and 81 fulfil the requirements for an adequate translation initiation site, according to Kozak⁸.

Although the dibasic (Arg-Arg) sequence in positions 13 and 14 of the predicted sequence represents a potential post-translational cleavage site, the amino-acid composition of purified CNTF (Table 1) does not favour such a cleavage: the amino acids phenylalanine, arginine and alanine present in the N-terminal region are not reduced relative to other amino acids that are absent from this region (for example, isoleucine). Moreover, the predicted relative molecular mass (M_r) of 22,800 (22.8K), corresponding to the 200-amino-acid sequence is in complete agreement with that estimated from PAGE analysis (22.5K) (ref. 6). Thus, the amino-acid sequence of CNTF shows the features of a cytosolic protein; that is, no signal peptide, no consensus sequences for glycosylation and only one cysteine residue at position 17. Comparison of the determined CNTF sequence with those of the PIR and EMBL databases did not reveal significant similarities with any other known protein. In

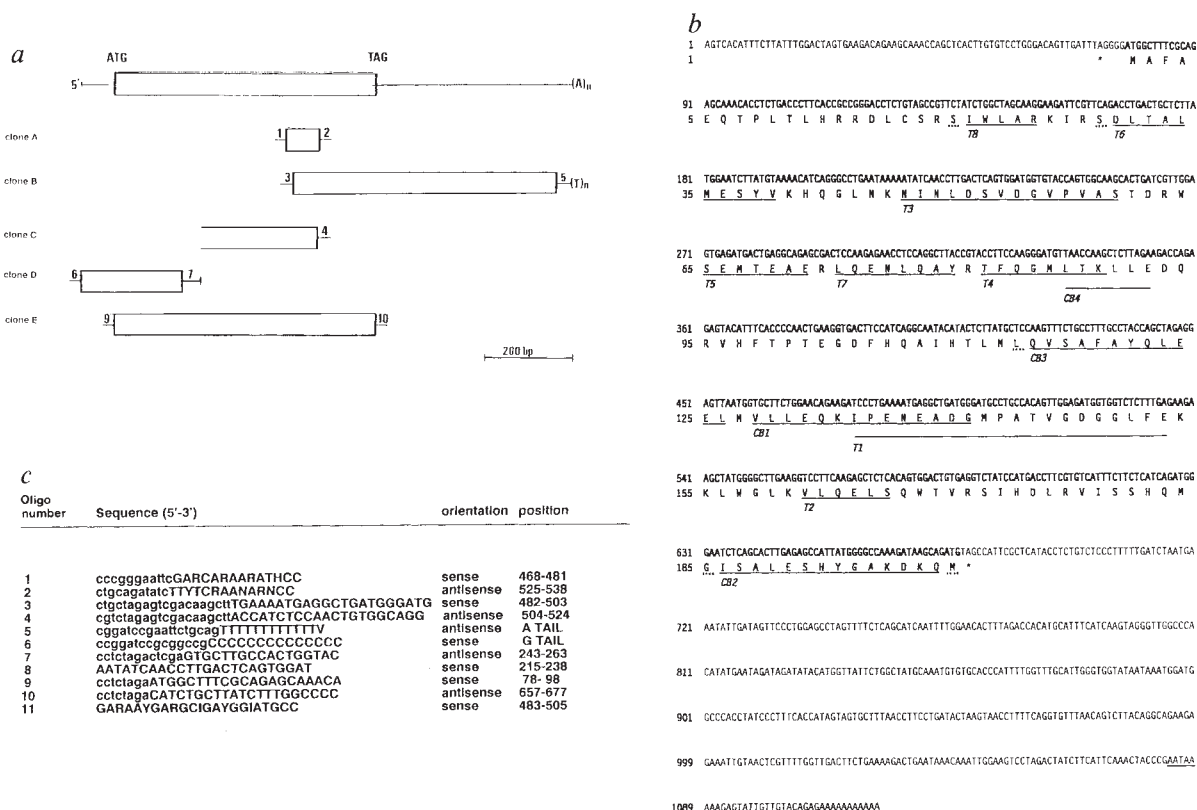


FIG. 1 **a**, Complementary DNA cloning, and **b**, nucleotide and deduced amino-acid sequence of CNTF. The oligonucleotides used as primers are shown in **c** and their corresponding positions in the nucleotide sequence in **b**. Multiple cloning sites are indicated as lower-case letters. In the case of degenerated codons we used IUPAC symbols. Underlined amino acids in **b** correspond to the peptide sequences obtained from tryptic (T1-T8) and cyanogen bromide (CBI-4) fragments of CNTF. At the nucleotide level, sequences corresponding to the coding region are bold-faced and the polyadenylation signal sequence is underlined.

METHODS. Cultured rat astrocytes were grown for 3 weeks as described²⁰. One day after final subculturing, total RNA was extracted from the cells²¹. Complementary DNA was synthesized using oligonucleotide 5 and reverse transcriptase of the Moloney murine leukaemia virus (Bethesda Research Laboratories). The first strand of cDNA served as a template for amplification of specific segments of CNTF using PCR²². Clone A was generated using

the degenerated primer-oligos 1 and 2; the PCR product was identified with oligonucleotide 11, subcloned and sequenced by the dideoxynucleotide chain-termination method²³. The nucleotide sequence thus obtained was used to synthesize primers 3 and 4 for amplification of cDNA ends according to Frohmann *et al.*²⁴ The cDNAs obtained were subcloned into the Bluescript SK+ vector (Stratagene) and sequenced (clones B and C). Oligonucleotide 7 was derived from the sequence of a genomic clone (Carroll, unpublished data). This primer, together with oligonucleotide 6 was used to create clone D using essentially the same protocol as for clone C, except that we performed a G-tailing procedure instead of T-tailing to generate 5' clones²⁵. The inserts of several clones were sequenced and shown to be identical. PCR with newly synthesized cDNA from astrocytes and oligonucleotides 9 and 10 revealed a cDNA clone covering the entire coding region. The cDNA thus obtained (Fig. 1a, clone E) was used for expression in eukaryotic cells.

particular, there was no homology with nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF)⁹ or fibroblast growth factor (FGF) and purpurin, the survival activities of which overlap to some extent with those of CNTF^{10,11}.

The likelihood that CNTF is a cytoplasmic protein is supported by the observation that expression of a cDNA clone in HeLa cells (Fig. 2) resulted in active CNTF being expressed but not being released into the culture medium. We cannot exclude however, that CNTF is secreted by any unconventional release mechanisms, as expression of clone E (Fig. 1a) reveals a C-terminally extended product. CNTF, rather, seems to be a molecule similar to FGF or interleukin-1 (IL-1) both of which exert profound effects on cells but are cytoplasmic proteins. For FGF, no release mechanism has been established¹². By contrast, IL-1 has been demonstrated to be released from stimulated macrophages by an unconventional mechanism after cleavage by a specific enzyme (convertase)¹³. Of particular interest is the recent finding that macromolecules may be exported from the yeast cytosol by carriers that show structural homologies with the multidrug-resistance glycoprotein in mammalian cells¹⁴.

Whether this glycoprotein can also act as a protein carrier in mammalian cells remains to be established.

Northern blot analysis of the distribution of CNTF messenger RNA in tissues of adult rat revealed a single band ~1.2 kilobases (kb) in size. By far the strongest signal was present in northern blots of the sciatic nerve and a faint band was present in extracts of the spinal cord. But there was no detectable signal in mRNA of muscle and skin; that is, <2 pg of CNTF mRNA in 50 µg and 30 µg of total RNA respectively. The low levels of CNTF mRNA in muscle and skin indicate that the large amount of CNTF present in the sciatic nerve does not represent CNTF transported retrogradely from the periphery—as is the case for NGF—but represents locally synthesized CNTF. Moreover, the developmental time course of CNTF mRNA expression differs from that of NGF¹⁵. CNTF mRNA was undetectable in sciatic nerves of newborn rats, only becoming apparent by day 4 (Fig. 3b). The developmental time course of CNTF mRNA expression indicates that CNTF is not involved in the regulation of neuronal survival in the perinatal period, because target-regulated neuronal cell death^{16,17} is already over by the time the increase in CNTF synthesis begins.

CNTF differs from the known neurotrophic factors NGF and BDNF by the absence of a known constitutive release mechanism, by the time course of its expression during development

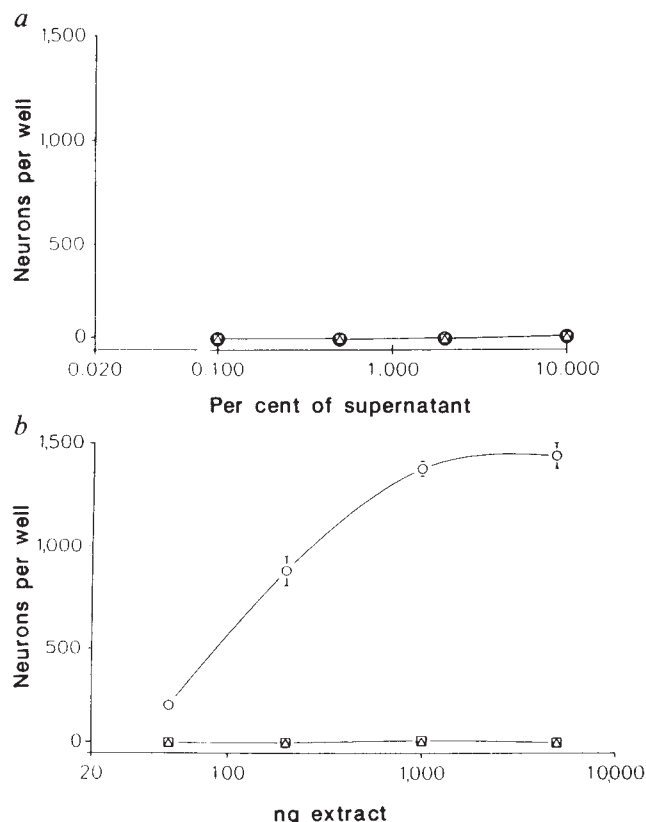


FIG. 2 Survival of cultured E8 chick ciliary neurons in the presence of supernatants and extracts of transfected HeLa cells. Embryonic day-8 ciliary neurons were grown in the presence of supernatants (a) and extracts (b) of HeLa cells transfected with plasmid without insert (Δ) and plasmid containing CNTF cDNA clone E in sense ($\circ$) and antisense orientation ($\square$). METHODS. An expression vector with cytomegalo-promotor (gift of David Russell, Dallas) was used for subcloning a mature CNTF clone (Fig. 1a, clone E) in both orientations. HeLa cells were used for transfections, as no basal expression of survival-promoting activity for embryonic chick ciliary neurons could be detected in these cells. Each culture dish (100-mm diameter) was transfected with 10 µg of vector by the DEAE-Dextran method²⁶. After 48 h in culture the supernatants were removed, the cells washed three times with cold PBS buffer and lysed in a 5 mM phosphate buffer containing 30 mM NaCl (pH 7.0). After ultracentrifugation (100,000 g, 30 min) of the lysate, protein concentrations were determined and different concentrations of the supernatants were added to cultured E8-ciliary neurons. Surviving neurons were counted after 24 h of culture as described previously⁵. Each point shows the mean of three determinations; bars represent the standard errors.

TABLE 1 Amino-acid composition of CNTF

	Purified CNTF	Predicted from cDNA
Asx	17.1	16
Thr	9.9	12
Ser	9.7	14
Glx	30.5	30
Pro	n.d.	5
Gly	11.7	11
Ala	14.4	14
Val	11.9	11
Met	7.3	9
Ile	7.7	8
Leu	24.5	26
Tyr	4.3	4
Phe	6.4	6
His	6.2	8
Lys	10.2	10
Arg	11.1	11
Cys	n.d.	1
Trp	n.d.	4

Amino-acid composition of CNTF. The amino-acid composition of purified CNTF corresponds to that predicted from the cDNA sequence. Purification and cleavage of CNTF: CNTF was purified as described⁶ but, in addition, after electroelution from preparative polyacrylamide gels, CNTF was applied to a C4-Widepore RP column (Baker, 7.75 × 100 mm) and eluted with 0.1% trifluoroacetic acid and a gradient of acetonitrile. The biologically active protein eluted in one peak at 50–55% acetonitrile. Two-dimensional gel analysis showed that this protein migrated as a single spot⁶. CNTF obtained by this method was concentrated in a Speedvac-concentrator to a final volume of 50 µl, diluted to 1 ml with HPLC-grade water and concentrated again to 200 µl. For cyanogen bromide cleavage, formic acid (final concentration 70% v/v) and cyanogen bromide (10% w/v) were added to 30 µg of purified CNTF. After 3 h at room temperature, 500 µl H₂O was added and the material concentrated to 50 µl and applied immediately to the same reverse phase-HPLC column. For tryptic cleavage, 30 µg HPLC-purified CNTF were dried, redissolved in 50 µl 0.1 M Tris HCl (pH 8.0) containing 10 mM CaCl₂ and 3 µg tosyl phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma), and incubated overnight at 37 °C. The resulting fragments were loaded on a C4-RP column (Baker) and eluted using the same conditions (flow rate 1 ml min⁻¹, gradient 0–60% acetonitrile in 60 min, monitoring at 214 nm). Peaks were collected manually. The amino-acid sequences of the peptides were determined²⁷ by use of automated gas-phase sequencing (Applied Biosystems 470A, 477A). The amino-acid composition of purified CNTF was determined after gas-phase hydrolysis¹⁸ of 5 µg of CNTF on a Beckman 6300 amino-acid analyser. n.d., Not done.

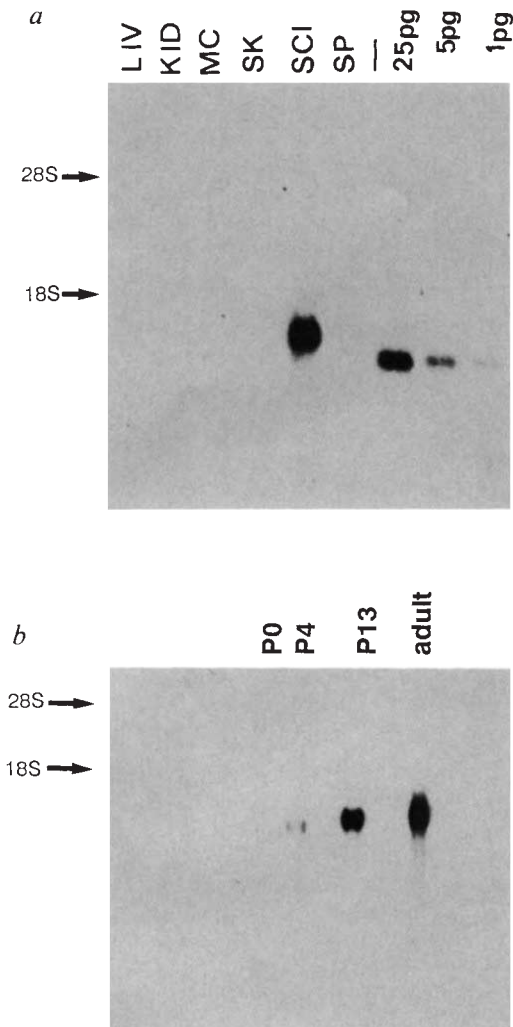


FIG. 3. Northern blot analysis of CNTF mRNA in tissues of the adult rat (a) and in rat sciatic nerve during development (b). The abbreviations used are: LIV, liver; KID, kidney; MC, muscle; SK, skin; SCI, sciatic nerve; SP, spinal cord; P0, newborn rats; P4, 4-day-old rats; P13, 13-day-old rats. METHODS. RNA was extracted from various rat tissues using the guanidinium thiocyanate extraction method²⁷. Total RNA (30 µg, except RNA from muscle which was 50 µg) was glyoxylated and electrophoresed through a 1.2% agarose gel²⁸. In b, 25 µg total RNA were loaded per lane. Known amounts of a 847-bp CNTF transcript (synthesized *in vitro* using the riboprobe system, PROMEGA) were also co-electrophoresed in separate lanes to permit quantification of CNTF mRNA in the samples. Following electrophoresis, RNA was vacuum-blotted to nylon filters (Hybond-N, Amersham) and the filters were hybridized at 50 °C in 50% formamide²⁸ using a randomly primed ³²P-labelled cDNA probe²⁹ covering the coding region of CNTF (600 bp). The filters were subsequently washed, exposed for 60 h to X-ray films and the autoradiograph was photographed.

and by its regional distribution. It may be that CNTF has a physiological role as a differentiation factor, its neurotrophic function possibly only being exerted under pathophysiological conditions rather than during embryonic development. □

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Alternative splicing directs the expression of two D₂ dopamine receptor isoforms

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DOPAMINE receptors are classified into D₁ and D₂ subtypes on the basis of their pharmacological properties and the intracellular responses they mediate¹. The cerebral D₂ dopamine receptor is the target of drugs used to alleviate the main symptoms of schizophrenia^{2–4}. Although it is considered to be a single molecular entity, there is evidence that multiple D₂-receptor subtypes exist^{5,6}. A complementary DNA encoding a D₂ receptor has recently been cloned⁷ and the deduced 415-amino-acid sequence indicates that it belongs to the large superfamily of receptors coupled to G proteins, and that its topology consists of seven transmembrane domains. In this family, the genes are frequently without introns and each is believed to encode a unique polypeptide product. Here we show that the gene for the D₂ receptor produces two receptor isoforms by alternative messenger RNA splicing, providing a route to receptor diversity in this family. One isoform corresponds to the D₂₍₄₁₅₎ receptor⁷, but the second contains an additional sequence encoding a 29-amino-acid fragment, defining a novel D₂₍₄₄₄₎ receptor isoform. Expression of the two isoforms is tissue-specific, and both are regulated by guanyl nucleotides. As the extra sequence is located within a putative cytoplasmic loop that binds to G proteins, the two isoforms might interact with different G proteins and thereby initiate distinct intracellular signals.

Synthetic oligonucleotides encompassing the putative transmembrane regions I and II and first cytoplasmic loop of the D₂₍₄₁₅₎ receptor⁷ were used to screen a rat brain cDNA library (Fig. 1). Out of six positive clones, restriction analysis and sequencing revealed two types of cDNAs: three clones corresponding to the D₂₍₄₁₅₎ receptor⁷, and three presenting an additional 87-base-pair (bp) sequence within the putative third cytoplasmic loop. This sequence has not previously been reported and encodes a deduced 29-amino-acid fragment, yielding a

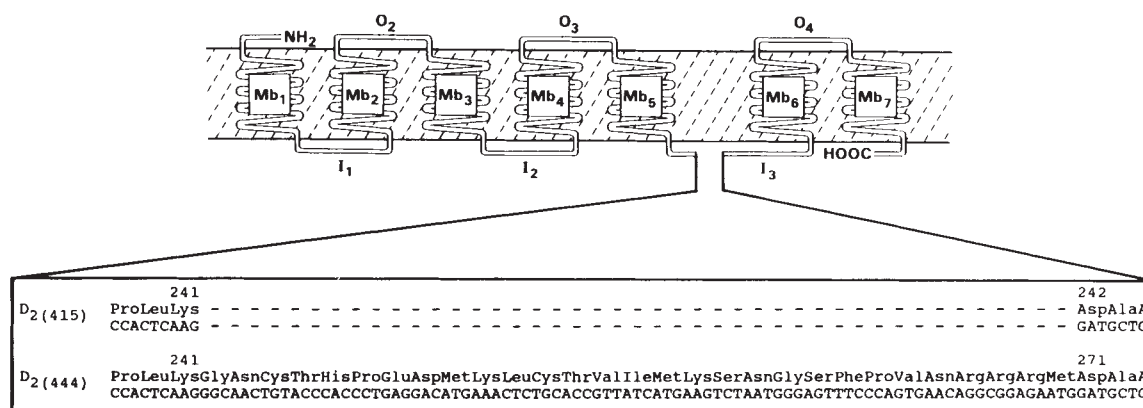


FIG. 1 Schematic representation of the structure of two isoforms of the D_2 dopamine receptor. These two isoforms, termed $D_{2(415)}$ and $D_{2(444)}$, differ in that the latter has a predicted 29-amino-acid sequence located within the putative third cytoplasmic loop between Lys 241 and Asp 242 (nucleotides 723 and 724). Note the presence of two potential glycosylation sites at Asn 243 and Asn 260. Otherwise the nucleotide sequence of the two clones is identical to that established by Bunzow *et al.*⁷.

METHODS. Overlapping oligonucleotides were synthesized (Clontech) from the sequence of D_2 dopamine receptor cDNA (nucleotides 146–233 in ref. 7), radiolabelled by 'filling reaction' using *Escherichia coli* DNA polymerase I (Klenow fragment; Boehringer) and used to screen an oligo(dT)-primed cDNA library constructed in λ gt10 (refs 20 and 21) from total rat brain

poly(A)⁺ RNA^{22,23}. After transfer of the phage onto nitrocellulose filters (BA 85; Schleicher & Schuell), they were hybridized at 42 °C for 16 h in a buffer (6 × SSC, 0.05% sodium pyrophosphate, 8 mM Tris-HCl buffer, pH 7.4, 20% formamide, 1 × Denhardt's solution, 100 μ g ml⁻¹ yeast transfer RNA and 20 μ g ml⁻¹ salmon sperm DNA) containing 5×10^5 d.p.m. of the synthetic probe per ml. Filters were washed twice for 20 min at 25 °C in 2 × SSC, 0.05% sodium pyrophosphate, 0.1% SDS and, twice for 30 min at 37 °C in 0.2 × SSC, 0.1% SDS, 0.05% sodium pyrophosphate. *Eco*RI inserts of positive clones were subcloned in M13 mp18 or mp19 vectors (Boehringer) for sequencing²⁴ and in pGEM-4Z (Promega Biotec) for restriction analysis and preparation of fragments for use as probes.

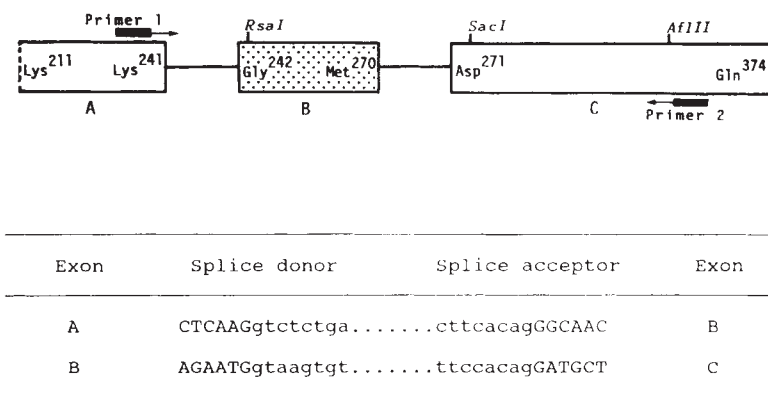
protein with a total of 444 amino acids: the $D_{2(444)}$ receptor (Fig. 1). The sequences otherwise are 100% homologous in their coding regions and with the cDNA previously described⁷.

We analysed rat genomic DNA to confirm that the $D_{2(415)}$ and $D_{2(444)}$ receptors are products of the same gene. First, Southern blot analysis indicated the presence of unique *Bam*HI and *Eco*RI restriction fragments, of 7.5 and 4.5 kilobases (kb) respectively, when probed with the *Sac*I-*Afl*III fragment (Fig. 2), which corresponds to a part of the third cytoplasmic loop common to both $D_{2(415)}$ and $D_{2(444)}$ receptors (data not shown). We also screened a genomic library with a *Bam*HI-*Bgl*II probe containing the full coding sequence of the cDNA for the $D_{2(444)}$ receptor (nucleotides 4 to 1,319), and then selected 21 clones out of 10^6 that were either identical or overlapping, using restriction enzyme digestion and Southern blot analysis. All of these clones hybridized strongly to an *Rsa*I-*Sac*I probe, corresponding mainly to the 29-amino-acid fragment characteristic of the $D_{2(444)}$ receptor (Fig. 2). Partial sequencing of these clones revealed the complex organization of the gene: it is at least 12 kb

long and contains a minimum of six introns, including five in the coding region. Four introns are located at nucleotide positions -33, 286, 533 and 1,142 of the cDNA for the $D_{2(415)}$ receptor, the last one of which has been reported⁷. It is noteworthy that the 87-bp sequence characteristic of the $D_{2(444)}$ receptor is flanked by two other introns, indicating that the two isoforms of the D_2 receptor are products of the same gene, generated by alternative mRNA splicing of the 87-bp exon (Fig. 2). Although all other splice donor and acceptor sequences consist of frequently occurring sequences⁸, this is not the case for the splice donor sequence in the intron upstream of the 87-bp exon, which may account for its alternative recognition by the splicing machinery, possibly itself a regulated mechanism. As the 87-bp exon does not contain any splicing consensus sequence, the $D_{2(444)}$ receptor mRNA does not represent an immature mRNA precursor of the shorter form.

Both isoform mRNAs seem to be expressed in all areas of the brain and pituitary in which the D_2 receptor is found^{4,9}, as indicated by northern analysis using either a probe selective for

FIG. 2 Diagram of the D_2 receptor gene showing the alternative splicing sites. Indicated are the positions of introns (lines) and exons (boxes), including the 87-bp exon selectively translated in the case of the $D_{2(444)}$ receptor isoform (shaded box). The corresponding amino acids flanking the exons are also indicated and numbered according to the sequence of the $D_{2(444)}$ receptor isoform. Restriction sites and primers refer to those used to prepare probes for northern blotting and to initiate the PCR as shown in Fig. 3. **METHODS.** A rat genomic library (from Dr A. M. Duchemin) constructed in Charon 4A phage from DNA partially digested with *Eco*RI was screened (10^6 phage) using as probe a ³²P-labelled nick-translated 1.3-kb *Bam*HI-*Bgl*II fragment of the $D_{2(444)}$ receptor cDNA. Nitrocellulose filters with transferred phage were hybridized for 16 h at 42 °C in 40% formamide, 10% dextran sulphate, 4 × SSC, 8 mM Tris-HCl, pH 7.4, 1 × Denhardt's, 20 μ g ml⁻¹ yeast tRNA, 20 μ g ml⁻¹ salmon sperm DNA, 0.1% SDS, using probe at 5×10^5 d.p.m. ml⁻¹. The filters were washed as described in the legend to Fig. 1, except that the last washing was for 30 min at 50 °C. In positive clones, *Eco*RI restriction generated fragments 0.8, 4.5 and 5.5 kb long, all of which hybridized to the *Bam*HI-*Bgl*II probe. The 4.5-kb fragment also hybridized



to a probe prepared by ³²P-nick translation of the *Rsa*I-*Sac*I fragment of $D_{2(444)}$ receptor cDNA, whereas the other two did not. This fragment was subcloned in M13 mp18 and sequenced using primers located around and within the 87-bp sequence.

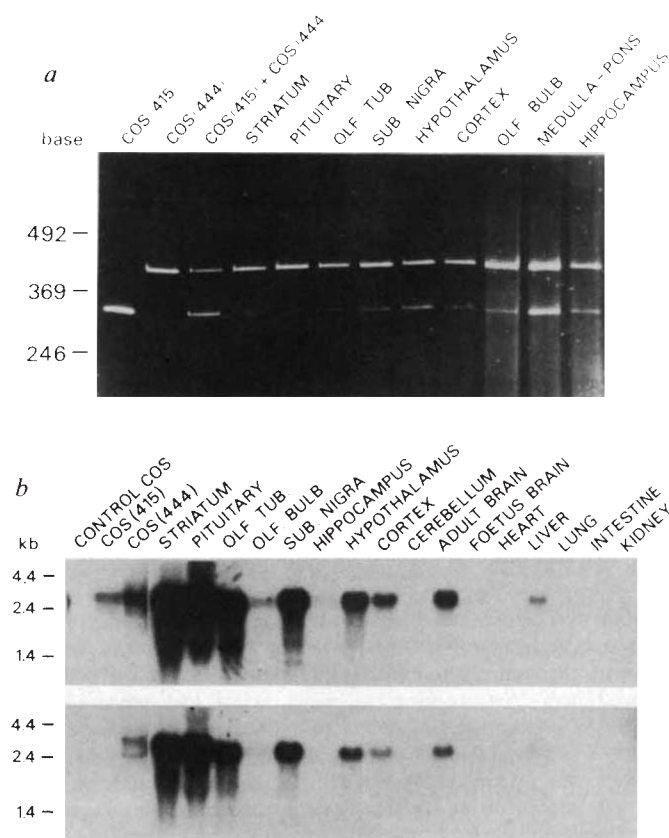


FIG. 3 Characterization of two D_2 dopamine receptor mRNAs. *a*, Northern blot analysis of total RNA from COS-7 cells (10 μ g) or poly(A)⁺ mRNA from rat tissues (5 μ g), using as probes either a 241-bp *SacI*-*AflIII* fragment (top) corresponding to a common sequence of the two isoforms or a 95-bp *RsaI*-*SacI* fragment (bottom; different blots from the same samples) corresponding mainly to the alternative exon (Fig. 2). *b*, Electrophoresis of PCR products from RNA using two primers flanking most of the putative third cytoplasmic loop (Fig. 2). The 397- and 310-base products correspond to the $D_{2(444)}$ and $D_{2(415)}$ receptor mRNAs, respectively. Abbreviations: COS(415) and COS(444), COS-7 cells transfected with $D_{2(415)}$ and $D_{2(444)}$ receptor cDNAs respectively; olf. tub., olfactory tubercle; olf. bulb., olfactory bulb; sub. nigra, substantia nigra; cortex, cerebral cortex; foetus brain, brain from gestational day-15 foetus; intestine, duodenum plus ileum. **METHODS.** Total RNA was extracted²² and poly(A)⁺ mRNA prepared²³ from tissues of male Wistar rats (except for pituitaries, which were from oestradiol-treated females²⁵) and COS-7 cells. *XhoI*-*SacI* fragments of recombinant pGEM-4Z containing $D_{2(415)}$ or $D_{2(444)}$ cDNAs were ligated into the *XhoI* restriction site of the pMAMneo inducible expression vector (Clontech). These constructs (30 μ g) were transfected²⁶ into mouse COS-7 cells (from Dr J. J. R  mi). Transformed cells were selected in 500 μ g ml⁻¹ G418 and 48 h after transfection, expression was induced by a 12–24 h treatment with 10 μ M dexamethasone. For northern blotting, denatured RNAs were electrophoresed in 1% agarose/formaldehyde gels, blotted onto nitrocellulose paper and baked for 2 h at 80 $^{\circ}$ C. Membranes were hybridized overnight at 42 $^{\circ}$ C in 40% formamide, 10 $\times$ Denhardt's, 50 mM Tris-HCl, pH 7.4, 4 $\times$ SSC, 0.1% sodium pyrophosphate, 1% SDS, 100 μ g ml⁻¹ salmon sperm DNA and 50 μ g ml⁻¹ yeast tRNA containing a ³²P-labelled nick-translated fragment at 5 $\times$ 10⁶ d.p.m. ml⁻¹. For PCR experiments, a single-strand cDNA was synthesized using AMV reverse transcriptase (20 U; Boehringer), primer 2 (5'-GCTTTCTGCGGCTCATCGTCTTAA; Eurogentech) and total RNA (from COS-7 cells containing identical amounts of specific mRNA, as assessed by northern blotting) or 1–6 μ g poly(A)⁺ mRNA (rat tissues). This template was amplified¹², using primer 1 (5'-TTCAGAGCCAACCTGAAGACACCA) and primer 2 (80 nM each) for 35 cycles (92 $^{\circ}$ C, 54 $^{\circ}$ C and 72 $^{\circ}$ C, for 1 min each), with 2.5 U *Taq* DNA polymerase (Perkin Elmer Cetus); 10–20% of PCR products were run on 6% polyacrylamide-urea gel which was stained in ethidium bromide and scanned.

the $D_{2(444)}$ receptor mRNA, or a probe hybridizing to both mRNAs (Fig. 3). In all these tissues, the first probe generated a signal, indicating unambiguously that the $D_{2(444)}$ receptor mRNA was expressed, and its intensity grossly paralleled the relative abundance of D_2 receptors⁹. Interestingly, a faint but distinct signal was also detected with mRNAs from liver, a tissue in which D_2 receptor may be expressed prenatally¹⁰. In all these tissues, the second probe also generated a signal of apparently parallel relative intensity, but we could not establish from it whether the $D_{2(415)}$ receptor mRNA was also expressed, or its relative abundance. But, using the polymerase chain reaction (PCR)^{11,12}, we clearly identified the two mRNA isoforms in brain and pituitary from two bands of the predicted sizes (Fig. 3) that hybridized with the *SacI*-*AflIII* probe (data not shown). The $D_{2(415)}$ receptor mRNA seems to be the least abundant in all regions tested, representing 2% in pituitary, 12% in olfactory

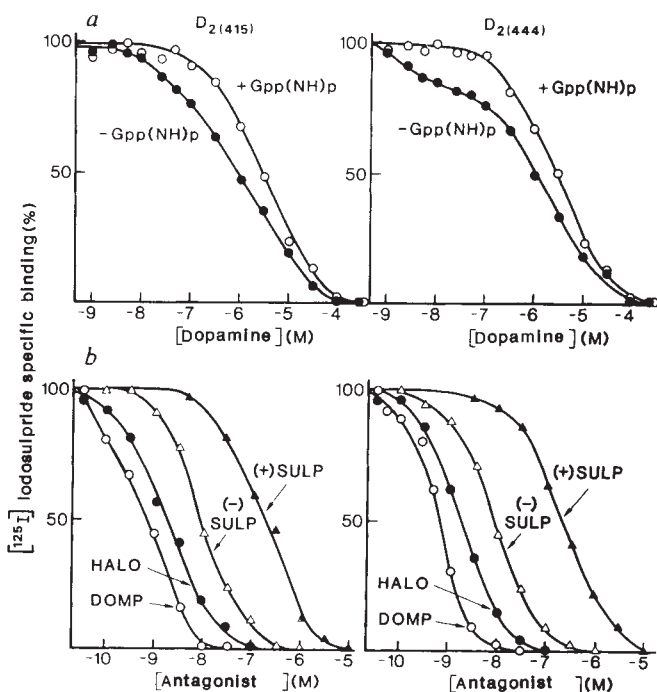


FIG. 4 [¹²⁵I]iodosulpride binding to the two D_2 receptor isoforms in transfected COS-7 cells. Specific binding of 0.4 nM [¹²⁵I]iodosulpride (about 2,000 Ci mmol⁻¹; Amersham) represented 100–150 and 150–200 fmol per mg protein in membranes of COS(415) and COS(444) cells respectively. *a*, Effect of Gpp(NH)p, a guanylyl nucleotide, on the dopamine inhibition of [¹²⁵I]iodosulpride binding to $D_{2(415)}$ and $D_{2(444)}$ receptors. In the absence of Gpp(NH)p, dopamine inhibited the specific [¹²⁵I]iodosulpride binding in a biphasic manner, giving the following IC₅₀ values and relative proportions of sites (in parentheses): 120 $\pm$ 40 nM (46%) and 6,500 $\pm$ 2,900 nM with the $D_{2(415)}$ receptor; 4.7 $\pm$ 2.8 nM (22%) and 1,900 $\pm$ 420 nM with the $D_{2(444)}$ receptor. In the presence of 0.1 mM Gpp(NH)p, only one site could be detected with IC₅₀ values of 2,290 $\pm$ 300 nM and 2,490 $\pm$ 260 nM for the $D_{2(415)}$ and $D_{2(444)}$ receptors respectively. Means from 3 separate experiments. *b*, Inhibition of specific [¹²⁵I]iodosulpride binding by various dopaminergic antagonists gave similar IC₅₀ values (nM) for the $D_{2(415)}$ and $D_{2(444)}$ receptor isoforms respectively: 0.64 $\pm$ 0.10 and 0.61 $\pm$ 0.06 with domperidone (DOMP); 1.3 $\pm$ 0.1 and 1.3 $\pm$ 0.2 with haloperidol (HAL); 9.1 $\pm$ 0.5 and 7.9 $\pm$ 0.3 with (-)sulpride ((-)SULP); 190 $\pm$ 30 and 170 $\pm$ 20 with (+)sulpride ((+)SULP). Values are means from 2–4 separate experiments. **METHODS.** COS-7 cells were scrapped, collected by centrifugation at 1,500g for 5 min and washed in 15 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂. After cell disruption, membranes were isolated by centrifugation (twice at 25,000g for 10 min) in the same buffer. Binding was assayed in 50 mM Tris-HCl pH 7.4 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 5.6 mM ascorbic acid and 0.1 mM 8-hydroxyquinoline⁹. Non-specific binding measured in the presence of 1 μ M (-)sulpride typically represented 20–25% of total binding. Curves were analysed by computerized nonlinear regression using a one- or a two-site model²⁵.

bulb, 20–25% in striatum, olfactory tubercle, substantia nigra, cortex and hippocampus, 40–45% in hypothalamus and pons-medulla. This indicates that the relative level of expression of the two isoforms is tissue-specific.

When expressed in COS-7 cells, which normally lack D₂ receptors, the two receptor isoforms displayed similar pharmacology when studied using [¹²⁵I]iodosulpride, a highly selective D₂ receptor ligand⁹ (Fig. 4). This agrees with the idea, derived from site-directed mutagenesis and chimaera construction, that the ligand-binding domain for this family of receptors resides in the transmembrane segments^{13,14}, which are identical in the two isoforms. It should be emphasized that neither the D₂₍₄₁₅₎ nor the D₂₍₄₄₄₎ receptor display the high affinity for benzamide derivatives which is characteristic of a putative D₂ receptor subtype^{5,6}. Both isoforms are apparently coupled to a G protein or proteins in the COS-7 cells, as judged from the modulation of dopamine affinity by a guanyl nucleotide (Fig. 4); this confirms that the absence of this feature in the D₂₍₄₁₅₎ receptor when it is expressed in L cells results from the absence of a suitable G protein, presumably a G_i protein, in the recipient cell⁷.

Hence a sizeable deletion in the third cytoplasmic loop, thought to be the domain through which these receptors couple to G proteins, does not prevent coupling; this is consistent with results from deletion mutagenesis¹³ and limited trypsinization in β -adrenergic receptors¹⁵. The significant variation in size and sequence of this loop among receptors of this family may reflect their selective interactions with members of the G-protein family, whose diversity probably results both from multiple genes and alternative splicing¹⁶. The slightly different pattern of Gpp(NH)p modulation observed with the two receptor isoforms in COS-7 cells (Fig. 4) could reflect either their interaction with different G proteins, or a different interaction with the same G protein.

For neurons, which are long-lived cells that have lost their ability to replicate DNA, alternative splicing is a reversible post-transcriptional mechanism that enables different proteins to be rapidly generated in response to environmental stimuli¹⁷. The selective tissue expression of the D₂ receptor isoforms by this process reveals a new paradigm in the generation of diversity among members of an already large family and raises questions about the underlying mechanisms and functional implications of this process and their disturbance. For instance, an increased number of D₂ receptors have been detected in the brains of schizophrenic patients and/or following their long-term treatments with antipsychotics^{18,19}; it will be interesting to determine which isoforms are involved and whether these changes have aetiological or therapeutic implications. □

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Multiple D₂ dopamine receptors produced by alternative RNA splicing

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DOPAMINE receptors belong to a large class of neurotransmitter and hormone receptors that are linked to their signal transduction pathways through guanine nucleotide binding regulatory proteins (G proteins). Pharmacological, biochemical and physiological criteria have been used to define two subcategories of dopamine receptors¹ referred to as D₁ and D₂. D₁ receptors activate adenylyl cyclase² and are coupled with the G_s regulatory protein³. By contrast, activation of D₂ receptors results in various responses including inhibition of adenylyl cyclase⁴, inhibition of phosphatidylinositol turnover⁵, increase in K⁺ channel activity⁶ and inhibition of Ca²⁺ mobilization⁷. The G protein(s) linking the D₂ receptors to these responses have not been identified, although D₂ receptors have been shown to both copurify^{8,9} and functionally reconstitute^{10,11} with both G_i and G_o related proteins³. The diversity of responses elicited by D₂-receptor activation could reflect the existence of multiple D₂ receptor subtypes, the identification of which is facilitated by the recent cloning of a complementary DNA encoding a rat D₂ receptor¹². This receptor exhibits considerable amino-acid homology with other members of the G protein-coupled receptor superfamily^{12,13}. Here we report the identification and cloning of a cDNA encoding an RNA splice variant of the rat D₂ receptor cDNA¹². This cDNA codes for a receptor isoform which is predominantly expressed in the brain and contains an additional 29 amino acids in the third cytoplasmic loop, a region believed to be involved in G protein coupling.

To isolate cDNAs encoding dopamine receptor subtypes, we initially constructed a λ ZAP II cDNA library using mRNA purified from rat striatum, the region of the brain known to contain the highest levels of both D₁ and D₂ dopamine receptors¹. This library was screened with a mix of two 36-mer synthetic oligonucleotides, the sequences of which were derived from amino acids 352–363 of the rat D₂ receptor cDNA¹². This region corresponds to the sixth transmembrane-spanning domain and is known to exhibit very high homology among previously cloned G protein-coupled receptors¹³. From 1 × 10⁶ recombinants, 15 positive clones were isolated. Restriction analysis and partial sequence information indicated that 5 of these clones were related to the rat D₂ receptor cDNA reported previously¹². One of the clones containing an insert of 2.5 kilobases (kb) was completely sequenced and the nucleotide and deduced amino-acid sequence of this cDNA is shown in Fig. 1. The longest open reading frame in this cDNA codes for a 444 residue protein with a relative molecular mass (M_r) of

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-146 AGGGACGGCGGCCCGGACGGCTGCCGAGGGGCGGCGGTGCGTGGATGCGGCGGGAGCTGGAAGCC

-79 TCGAGCAGCGCGCCCTTCTTGCCCCGGGCGCCATATGGCTTGAAGACCGTGCCACCCAGTGGCCCCACTGCCCA

1 ATG GAT CCA CTG AAC CTG TCC TGG TAC GAT GAC GAT CTG GAG AGG CAG AAC TGG AGC CGG
Met Asp Pro Leu Asn Leu Ser Trp Tyr Asp Asp Asp Leu Glu Arg Gln Asn Trp Ser Arg 20

61 CCC TTC AAT GGG TCA GAA GGG AAG GCA GAC AGG CCC CAC TAC AAC TAC TAT GCC ATG CTG
Pro Phe Asn Gly Ser Glu Gly Lys Ala Asp Arg Leu Ile Val Ser Leu Ala Val Ala Met Leu 40

121 CTC ACC CTC CTC ATC TTT ATC ATC GTC TTT GGC AAT GTG CTG GTG TGC ATG GCT GTA TCC
Leu Thr Leu Leu Ile Phe Ile Ile Val Phe Gly Asn Val Leu Val Cys Met Ala Val Ser 60

181 CGA GAG AAG GCT TTG CAG ACC ACC ACC AAC TAC TTG ATA GTC AGC CTT GCT GTG GCT GAT
Arg Glu Lys Ala Leu Gln Thr Thr Thr Asn Tyr Leu Ile Val Ser Leu Ala Val Ala Met Leu 80

241 CTT CTG GTG GCC ACA CTG GTA ATG CCG TGG GTT GTC TAC CTG GAG GTG GTG GGT GAG TGG
Leu Leu Val Ala Thr Leu Val Met Pro Trp Val Val Tyr Leu Glu Val Val Gly Glu Trp 100

301 AAA TTC AGC AGG ATT CAC TGT GAC ATC TTT GTC ACT CTG GAT GTC ATG ATG TGC ACA GCA
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361 AGC ATC CTG AAC CTG TGT GGC ATC AGC ATT GAC AGG TAC ACA GCT GTG GCA ATG CCC ATG
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421 CTG TAT AAC ACA CGC TAC AGC TCC AAG CGC CGA GTT ACT GTC ATG ATT GCC ATT GTC TGG
Leu Tyr Lys Thr Leu Gln Thr Ser Ser Lys Arg Arg Val Thr Ser Val Thr Ser Phe Thr Val 160

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Val Leu Ser Phe Thr Ile Ser Cys Pro Leu Leu Phe Gly Leu Asn Asn Thr Asp Gln Asn 180

541 GAG TGT ATC ATT GCC AAC CCT GCC TTT GTG GTC TAC TCC TCC ATT GTC TCA TTC TAC GTG
Glu Tyr Lys Ile Ala Asn Thr Pro Ala Phe Val Val Tyr Ser Ser Val Thr Ser Phe Thr Val 200

601 CCC TTC ATC GTC ACT CTG CTG GTC TAT ATC AAA ATC TAC ATC GTC CTC CGG AAG CGC CGG
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661 AAG CGG GTC AAC ACC AAG CGC AGC AGT CGA GGT TTC AGA GCC AAC CTG AAG ACA CCA CTC
Lys Arg Val Asn Thr Lys Ser Ser Arg Ala Phe Thr Ser Arg Ala Asn Lys Thr Pro Leu 240

721 AAG GGC AAC TGT ACC CAC CCT GAG GAC ATG AAA CTC TGC ACC GTT ATC ATG AAG TGT AAT
Lys Gly Asn Cys Thr His Pro Glu Asp Met Lys Leu Cys Thr Val Ile Met Lys Ser Asn 260

781 GGG AGT TTC CCA GTG AAC AGG CGG AGA ATG GAT GCT GCC CGC CGA GGT CAG GAG CTG GAA
Gly Ser Phe Pro Val Asn Arg Arg Arg Met Asp Ala Ala Arg Arg Ala Gln Glu Leu Glu 280

841 ATG GAG ATG CTG TCA AGC ACC AGC CCC CCA GAG AGG ACC CGG TAT AGC CCC ATC CCT CCC
Met Glu Met Leu Ser Ser Thr Ser Pro Pro Glu Arg Thr Arg Tyr Ser Pro Ile Pro Pro 300

901 AGT CAC CAC CAG CTC ACT CTC CTT GAT CCA TCC CAC CAC GGC CTA CAT AGC AAC CCT GAC
Ser His His Gln Leu Thr Leu Pro Asp Pro Ser His His Gly Leu His Ser Asn Pro Asp 320

961 AGT CCT GCC AAA CCA GAG AAG AAT GGG CAC GCC AAG ATT GTC AAT CCC AGG ATT GCC AAG
Ser Pro Ala Lys Pro Leu Lys Asn Gly His Ala Lys Ile Val Asn Pro Arg Ile Ala Lys 340

1021 TTC TTT GAG ATC CAG ACC ATG CCC AAT GGC AAA ACC CGG ACC TCC CTT AAG ACG ATG ACG
Phe Phe Glu Ile Gln Thr Met Pro Asn Gly Lys Thr Arg Thr Ser Leu Lys Thr Met Ser 360

1081 CGC AGA AAG CTC TCC CAG CAG AAG GAG AAG AAA GCC ACT CAG ATG CTT GCC ATT GTT CTC
Arg Arg Lys Leu Ser Gln Gln Lys Glu Lys Lys Ala Thr Gln Met Leu Ala Ile Val Leu 380

1141 GGT GTG TTC ATC ATC TGC TGG CTG CCC TTC ATC ACG CAC ATC CTG AAT ATA CAG TGT
Gly Val Phe Ile Ile Cys Trp Leu Pro Phe Phe Ile Thr His Ile Leu Asn Ile His Cys 400

1201 GAT TGC AAC ATC CCA CCA GTC CTC TAC AGC GCC TTC ACA TGG CTG GGC TAT GTC AAC AGT
Asp Cys Asn Ile Pro Pro Val Leu Tyr Ser Ala Phe Thr Trp Leu Gly Tyr Val Asn Ser 420

1261 GCC CTG AAC CCC ATC ATC ACC ACC TTC AAC ATC GAG TTC CGC AAG GCC TTC ATG AAG
Ala Val Asn Pro Ile Ile Tyr Thr Thr Phe Asn Ile Glu Phe Arg Lys Ala Phe Met Lys 440

1321 ATC TTG CAC TGC TGAGTCTGCCCTTGCTGCACAGCAGTGTCTCCACCTCCCTGCCTATGACGCCAGACCTCAT
Ile Leu His Cys 444

1399 CCCTGCAAGCTGTGGGCAGAAAGGCCAGATGGACTTGGCTTCTCTGACCCTGCAGGCCCTGCAGTGTAGCTTGGCTCG

1481 ATGCCCCCTCTCTGCCACACACCTCATCTGCCAGGTAGGGCCAGGGAGACTGGTATCTTACCAGCTCTGGGGTTGGACC

1563 CATGGCTCAGGGCAGCTCACAGAGTGGCCCTCTCATATCCAGACCTGTCTCTTGGCACCAGATGACGGCCCTCTCTT

1645 GACCTTCTCTTGGGCACAGAACTAGCTCAGTGGTGGACACACCTGATCGTGGCTTGGCTGGCCCTTGGCTGCTGT

1727 GCCAGATCAGGTGGTGGGAGGAGCAACAGTTCTTACTTTATAGGAACACATAGGAAGAGGGAACACGCCAAGTCTCTC

1809 AGGCAACATCAGTGTGAGGAGACACATAAACACACAGGTAGCTCCATGGACCCAGAGAACTGAGGCTGAAAAATCTGTT

1891 TTCCACTCCAACCTAGTGTGAGTCCCTACTTTTCATAGCCATGGGTATTACTATGTCTACCTTGTATAGTATCCATGG

1973 GGTTCCTGTACCTTTTGGGGGAAAACTCTAATCTCAAGGGCCCCAAGAGAACTGTGAAGGAGAAATAGGCTGATCT

2055 CCCTCTACTCTCCAACCTCAACCTCTCTTGATATACCTTGGATGTATCCATTCCTCACAGAAATGCTGGCCAGTCAG

2137 GCCTTGGACCAAGTGTGGAGTTGAAGCTGGATGGTAACTTGGGGCTCTTTGGGGCTGGGGGGTGTGAACATCTCTCT

2219 CTTCATATCTCTCTCTCCAGTGCCTCTGCTTGAAGAGGCTGTGGATGGGCTGTGGAGTCTGTATACCATTTGGGCC

2301 TGGCCCTGAATGAGGAGGGGAGCTGCAGTTTGGAGGGTTCTGGGATCCAACCTCTGAACATCACTATACCTGACCAAAAC

2383 TAATAAAACCTTGACAAGAGTCAAAAAA
2404

FIG. 1 Nucleotide and deduced amino-acid sequence of the D_2 receptor cDNA clone. The 87-bp/29 amino-acid insertion sequence is indicated by underlining. The nucleotide sequence is numbered from the initiator Met and indicated at the left of each line. The amino-acid numbers are indicated at the right of each line. The single base differences in the 3' untranslated sequence are indicated by dots.

METHODS. Poly(A)⁺ RNA was isolated from rat striatal tissue using standard procedures²² and used to construct a cDNA library in the λ ZAP II vector (Stratagene). The resulting library contained 6.1×10^6 independent recombinants. 1×10^6 recombinants from the unamplified library were screened using the oligonucleotide 5'-GATGA-AGAAGGGCAGCCAGCAGATGATGAACACA(G)CC-3' radiolabelled using [γ -³²P]ATP and T4 polynucleotide kinase. Duplicate nitrocellulose filters were hybridized in 0.3 M NaCl/0.03 M sodium citrate ($2 \times$ SSC), 0.02 M Na₂HPO₄, 0.1% SDS, 0.2 mg ml⁻¹ salmon sperm DNA, and 4×10^6 d.p.m. ml⁻¹ ³²P-labelled probe for 18 h at 42 °C. High stringency washing of the filters was performed with $0.5 \times$ SSC and 0.1% SDS at 60 °C before autoradiography. Phage λ found to hybridize to the probe were subsequently plaque-purified. *In vivo* excision and rescue of the nested pBluescript plasmids from the λ ZAP II clones were performed using helper phage according to the Stratagene protocol. Nucleotide sequence analysis was performed using the Sanger dideoxy nucleotide chain termination method on denatured double-stranded plasmid templates with Sequenase (US Biochemical Corp.). Primers were synthetic oligonucleotides which were either vector-specific or derived from previous sequence information. In some cases a series of nested deletion mutants were constructed using the *Exo* III/Mung Bean nuclease procedure (Stratagene) before DNA sequencing.

50,887. The nucleotide and amino-acid sequence within this coding region is identical to the rat D_2 receptor cDNA previously reported with the notable exception of an additional 87 base pair (bp) sequence coding for a 29 amino-acid insertion between residues 241 and 242 (ref. 12). This is located within the predicted third cytoplasmic loop ~30 residues from the C terminus of the fifth transmembrane-spanning domain. In addition to this insertion sequence, and a slightly extended 5' untranslated sequence, we also noted five base differences within the 3' untranslated

region compared with the previously published sequence¹² (Fig. 1). Subsequent sequence analysis indicated that all five of the D_2 receptor-related cDNAs initially isolated from this library contained the identical 87-bp insertion sequence. The nucleotide sequences delineating the boundaries of this insertion sequence correspond with consensus exon sequences for RNA splice junctions¹⁴, indicating that this cDNA results from alternative RNA splicing. The alternative hypothesis, that the different cDNAs are derived from separate genes, is unlikely given the

observation that an oligonucleotide probe corresponding to an identical sequence in the two cDNAs (Fig. 2a) hybridizes to single DNA fragments on Southern blots of rat genomic DNA cut with a number of different restriction enzymes (data not shown).

To confirm the D₂-subtype identity of this longer cDNA clone and to determine if the 29 amino-acid insertion sequence results in a major alteration in the ligand binding properties of the D₂ receptor, the cDNA was inserted into the SV40 promoter-driven vector, pEUK-C1 (ref. 15), for expression in eukaryotic cells. The resulting plasmid (pEUK-D2L) was used to transiently transfect COS-7 cells by the calcium phosphate precipitation technique¹⁶. After 3 days, membranes were prepared and the binding of the D₂ dopaminergic antagonist [³H]methylspiperone was examined¹⁷. In three separate transfection experiments, [³H]methylspiperone bound to the membranes in a saturable fashion with high specific activity (~ 1 pmol mg⁻¹ protein) and an affinity (62.1 ± 2.1 pM) in good agreement with that found in the rat striatum¹. No specific binding activity was detected in COS-7 cells that had not been transfected with pEUK-D2L or transfected with the pEUK-C1 vector alone. The ability of a variety of dopaminergic ligands to compete for specific [³H]methylspiperone binding to transfected COS-7 cell membranes was also investigated in these experiments. The high affinity D₂-selective antagonist spiperone (36 ± 3.8 pM) is the most potent agent, followed by the nonselective dopaminergic antagonist (+)butaclamol (0.52 ± 0.01 nM), which is more than four orders of magnitude more potent than its inactive isomer, (-)butaclamol (>10 μ M). The D₂-selective antagonist (-)sulpiride (7.9 ± 0.42 nM) also exhibits high affinity whereas the D₁-selective antagonist SCH-23390 (0.41 ± 0.047 μ M) does not. The Hill coefficients for these antagonist competition curves do not differ significantly from unity. Dopamine is also able to inhibit completely [³H]methylspiperone binding ($K_i = 0.71 \pm 0.012$ μ M), although the competition curve is homogeneous

(Hill coefficient ~ 1) and not significantly affected by guanine nucleotides indicating the absence of appropriate G-protein coupling¹⁸ in the COS-7 cells. This rank order of potency as well as the absolute affinities (K_i) of the antagonists agree with those demonstrated for brain D₂ receptors¹, as well as those for the D₂ receptor encoded by the previously cloned cDNA¹². These preliminary experiments thus indicate that the insertion sequence does not appear to affect the basic ligand recognition properties of the D₂ receptor.

To verify the expression of the D₂ receptor variant containing the insertion sequence and determine the relative proportions of the two receptor isoforms, we subjected various rat tissues to northern blot analysis using an oligonucleotide probe to a consensus region (Fig. 2a) as well as an insert sequence-specific

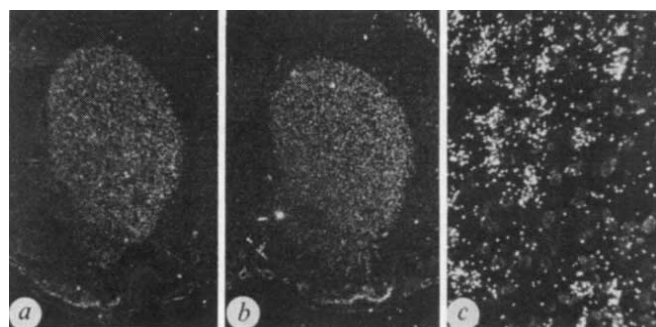


FIG. 2 Northern blot analysis of D₂ receptor transcripts in brain and other rat tissues. Each lane contained 2 μ g poly(A)⁺ RNA. Lanes: 1, total brain; 2, cerebellum; 3, cortex; 4, hippocampus; 5, olfactory bulb; 6, mesencephalon; 7, retina; 8, kidney; 9, striatum; 10, pituitary. The gel locations of the RNA size markers (kb) are indicated, a, Blots hybridized with an oligonucleotide derived from amino acids 10–25 (Fig. 1): 5'-TGACCCATTGAAGGGCCGGCTCCAGTTCTGCCTCTCCAGATCGTCATC-3'. b, Hybridization performed with an insert sequence oligonucleotide derived from amino acids 242–257 (Fig. 1): 5'-CATGATAACGGTGCAGAGTTTCATGCTCCTCAGGTGGGTACAGTTGCC-3'. This experiment was performed twice with similar results.

METHODS. Poly(A)⁺ RNA was isolated from rat tissues using standard procedures²² which included 2–3 passages over oligo(dT) cellulose. This procedure resulted in mRNA of high purity nearly devoid of 28S and 18S rRNA. After electrophoresis on 1% agarose plus 0.66 M formaldehyde gels and blotting, the filters were prehybridized in 4 $\times$ SSPE, 5 $\times$ Denhardt's solution, 50% formamide, 250 μ g ml⁻¹ yeast tRNA, 500 μ g ml⁻¹ sheared salmon sperm DNA, and 0.1% SDS for 16 h at 37 °C. The filter blots were then hybridized in the same solution for 18 h at 37 °C with 2×10^6 d.p.m. ml⁻¹ oligonucleotide probe radiolabelled with [α -³²P]ATP and terminal deoxytransferase. The blots were washed in 1 $\times$ SSPE and 0.1% SDS for 20 min four times at 56 °C and twice at room temperature before autoradiography.

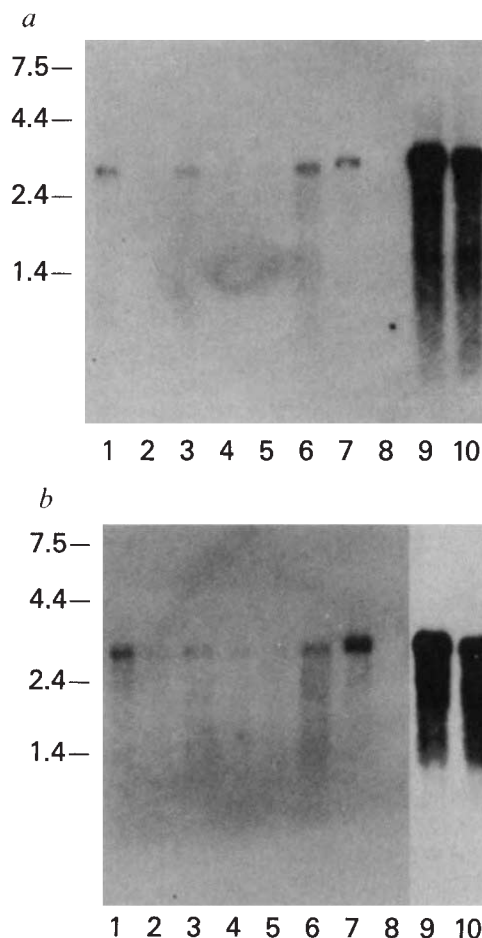


FIG. 3 Darkfield photomicrographs (silver grains appear white) of *in situ* hybridization histochemical (ISHH) localization of D₂ receptor mRNA in a coronal section of rat brain which includes the striatum. a, ISHH labelling using the ³⁵S-labelled oligonucleotide from Fig. 2a. b, ISHH labelling using the ³⁵S-labelled oligonucleotide from Fig. 2b. In both sections the labelling of cells is most dense in the striatum and olfactory tubercle. c, High power magnification of labelling from b showing localization of D₂ receptor mRNA in a subset of medium-sized neurons in the striatum. Approximately half of the medium-sized cells are labelled by this procedure.

METHODS. Coronal sections through the striatum were cut in a cryostat and adhered to glass slides that had been twice coated with gelatin. Sections were fixed in a 4% paraformaldehyde solution in 0.9% saline for 10 min, rinsed and incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% saline (pH 8) for 10 min, dehydrated in ethanol and defatted for 2 $\times$ 5 min in chloroform, rehydrated and air-dried. These sections were then hybridized with the ³⁵S-dATP tailed oligonucleotide probes and processed as previously described²³. Subsequently, the slides were dipped in NTB3 emulsion (diluted 1:1 with water) and exposed for 4–6 weeks, after which they were developed in D-19 developer for 2 min, fixed, rinsed, counterstained with thionin, dehydrated and coverslipped out of xylene.

probe (Fig. 2b). As shown in Fig. 2, the tissues expressing the highest levels of the 2.9-kb D_2 receptor mRNA are the striatum and pituitary. The retina shows a moderate abundance of mRNA with low levels being observed in the mesencephalon and cortex and trace quantities detected in the olfactory bulb and hippocampus. Little mRNA was found in the cerebellum and kidney. This tissue distribution corresponds closely to that previously determined for D_2 receptor expression¹. Of interest, however, is the fact that in all of the tissues examined, the amount of mRNA detected with the two probes is very similar (Fig. 2) and in no instance did the consensus probe detect greater quantities of mRNA.

To investigate further the relative distributions of the two mRNAs encoding the D_2 receptor isoforms we performed *in situ* hybridization analysis in the rat forebrain with the two oligonucleotide probes used in Fig. 2. Figure 3 shows darkfield photomicrographs of *in situ* hybridization histochemical localization of D_2 receptor mRNA in a coronal section of rat brain which includes the striatum. As can be seen, identical patterns of labelling were obtained using both the consensus region probe and the insert sequence probe (Fig. 3a, b). The highest labelling occurred in the striatal neurons where ~50% of the medium sized neurons were labelled (Fig. 3c). Larger sized neurons in the striatum also exhibited labelling (data not shown).

It is interesting that in Figs 2 and 3 there did not appear to be any difference in the levels of mRNA detected using the two oligonucleotide probes. If any tissue or brain area expressed mRNA containing the insertion sequence at a level equal to or less than the one lacking the insertion, then the consensus probe should detect mRNA levels that are at least twofold greater than those seen with the insert probe. These experiments thus indicate that not only is the longer D_2 receptor variant (which we propose designating D_{2L}) expressed in brain and other tissues, but in those areas which have been examined (especially the striatum), it appears to be the major isoform. Exploring this further, we rescreened our unamplified rat striatal cDNA library at high stringency using the full length D_{2L} receptor cDNA as a probe, which resulted in the isolation of ~24 D_2 -receptor-positive clones. Partial sequence analysis indicated that only three of these clones correspond to the previously isolated cDNA¹² encoding the shorter D_2 receptor (now designated D_{2S}). This indicates that the D_{2S} receptor is, in fact, expressed in the striatum, albeit in low abundance. Further experiments directed at determining the actual levels of the receptor proteins will be required to confirm this point.

With the exception of the visual opsins, the genes for the G protein-coupled receptor family have, in most instances, demonstrated a lack of introns within their coding sequences¹³ thus precluding the generation of receptor diversity through alternative RNA splicing. Recently, however, it has been determined that the serotonin 5HT_{1C} (B.J. Hoffmann, personal communication) and D_2 dopamine¹² receptors are encoded by genes that contain introns. Our data on the rat D_2 receptor now provides an example of G protein-coupled receptor isoforms generated through alternative RNA splicing. These isoforms, which are defined by the presence or absence of a 29-amino-acid sequence within the receptor protein, could arise either through the existence of a 'cassette exon' or through alternative internal acceptor or donor sites within the precursor mRNA¹⁹. The isolation and sequencing of the rat D_2 receptor gene will be required to distinguish among these possibilities. The location of this optional amino-acid sequence is particularly intriguing as it occurs within the predicted third cytoplasmic loop of the receptor¹². Recent mutagenesis studies using the β_2 -adrenergic catecholamine receptor have indicated that this region is involved in G protein-receptor coupling^{20,21}. It is thus tempting to speculate that the two D_2 receptor isoforms are coupled to different G proteins thus resulting in the diversity of responses associated with D_2 receptor activation⁴⁻⁷. Further work involving the stable expression of the two D_2 receptor isoforms in cells

exhibiting appropriate G protein-linked effector systems will be required to test this hypothesis. □

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Mechanism of antigen-driven selection in germinal centres

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THE high affinity of antibodies produced during responses to T-cell-dependent antigens is associated with somatic mutation in the variable region of the immunoglobulin¹⁻⁴. Indirect evidence indicates that: (1) this arises by a process of hypermutation, acting selectively on rearranged immunoglobulin variable-region genes, which is activated in centroblasts within germinal centres; and (2) centrocytes, the progeny of centroblasts, undergo selection on the basis of their ability to receive a positive signal from antigen⁵. We have now performed experiments analysing this selection process, and found that, on culture, centrocytes isolated from human tonsil kill themselves within a few hours by apoptosis⁶. This is not a feature of other tonsillar B cells. Centrocytes can be prevented from entering apoptosis if they are activated both through their receptors for antigen and a surface glycoprotein recognized by CD40 antibodies^{7,8}.

Immunohistological studies have shown that B cells undergo a marked phenotypic change when they participate in germinal-centre reactions⁸. The lack of surface IgD and CD39 antigen on either centroblasts or centrocytes was used to isolate human germinal-centre B cells by negative selection from suspensions of medium- and low-density tonsillar B cells (Table 1). The phenotype of the resulting CD39-IgD-negative fraction (Table 1) corresponds with that defined for germinal-centre B cells using immunohistology⁸. These cells show good viability at the end of the isolation procedure, carried out at 4 °C. When these cells were cultured at 37 °C they began to show signs of apoptosis (Fig. 1a). This process resulted in the loss of ~75% of the cells within 16 h, with no cells surviving 40 h (Table 2). By contrast, apoptosis was not a feature either of the medium-to-low density tonsillar B cells that expressed CD39 or IgD, or both, or of dense tonsillar B cells (Table 2). Similar results were obtained when germinal-centre cells were positively selected for

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expression of CD38 (Table 2). Apoptosis in the germinal-centre cell-enriched preparation was confirmed by: (1) finding the characteristic DNA fragmentation, caused by endonuclease activity which cuts DNA at intervals of about 180 bases⁶, using methods described previously⁹; and (2) identifying the morphological features of apoptosis, which include chromatin condensation with nuclear fragmentation, by both conventional (Fig. 1a) and electron microscopy⁶.

Follicular dendritic cells take up antigen, in the form of immune complex, and retain this for extended periods^{10,11}. These may provide a means for antigen-driven B-cell selection. A series of experiments were carried out to see if apoptosis in isolated germinal-centre cells could be prevented by exposing the cells to a range of antibodies to human immunoglobulins. None of these prevented apoptosis when added in soluble form to cultures. When polyspecific anti-immunoglobulin was conjugated to sheep red blood cells and added to cultures, however, it inhibited apoptosis (Table 3a, Fig. 1b). This protective effect was also obtained with anti-IgG, but not with other immunoglobulin class-specific antibodies bound to red cells (Table 3b). IgG was the only class of immunoglobulin expressed on a substantial proportion of isolated germinal-centre cells (Table 1).

Germinal-centre B cells were not prevented from entering apoptosis when they were cultured with a range of other agents that stimulate B cells. Among such agents tested were the recombinant cytokines, interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6

and IL-7, CD23 antibody, gamma and alpha interferon and supernatant from mixed lymphocyte reaction cultures (data not detailed). These agents were all added in concentration ranges known to be biologically active in control human B-cell cultures. By contrast, the addition of soluble CD40 antibody greatly reduced the number of cells entering apoptosis (Table 3a). Although anti-immunoglobulin and CD40 were effective in delaying the onset of apoptosis, neither agent used in isolation was able to sustain viability beyond 2 days of culture. Good survival after 4 days of culture was obtained when the two agents were added in combination (Table 3a). The CD40 effect could reflect a microenvironment-dependent signal.

The trigger for apoptosis in cultured germinal-centre cells is unclear. It is unlikely that the cells are killed by cytolytic lymphocytes, because apoptosis continues in the absence of extracellular divalent cations (data not shown). This was demonstrated using techniques described in ref. 12. Calcium, and to a lesser extent Mg^{2+} , are required for lymphocyte-mediated

TABLE 1 Phenotype of tonsil B cells

Antigen detected	B-cell fraction	
	Enriched for germinal-centre cells (sCD39 -ve and slgD -ve)	Depleted of germinal-centre cells (sCD39 +ve and/or slgD +ve)
sCD19	85 (79-95)	88 (84-98)
sCD10	74 (65-80)	22 (15-30)
sCD38	86 (81-92)	32 (23-50)
sCD39	0 (0-2)	53 (38-75)
sCD3	0 (0-0)	0 (0-0)
slgG	47 (28-52)	34 (24-43)
slgM	3 (1-16)	26 (24-37)
slgD	0 (0-0)	60 (54-70)
slgA	0 (0-0)	2 (1-4)
Alpha-NAE	5 (4-5)	3 (3-3)

The phenotype of tonsil B cells either enriched for germinal-centre cells by removal of cells binding CD39 antibody or anti-IgD, or both, or depleted of germinal-centre cells. The values shown are the median value for the percentage of cells positive, based on five experiments, with the range in parentheses. Tonsil B cells were prepared as previously described²⁴; all separation procedures were performed at 4 °C. High-density cells which lack the features of germinal-centre B cells were removed by centrifugation through a 60% Percoll gradient. The remaining cells were mixed with sheep red cells coated with CD39 antibody AC-2 (ref. 8) and red cells coated with sheep anti-human IgD. After 30 min, the non-rosetting, germinal-centre cell-enriched fraction was separated from the rosetting fraction using Ficoll-isopaque density gradient centrifugation. The surface (s) phenotype of the cells was determined using the direct antibody rosette technique²⁵. The nuclei of the cells were stained with 0.002% acridine orange to identify cells undergoing apoptosis and nonmononuclear cells. These were excluded from the counts. Alpha-naphthyl-acetate esterase (alpha-NAE) activity, used to identify macrophages, was revealed as described in ref. 26. The following monoclonal antibodies were used: CD19, an antigen present on all B cells (BU-12; ref. 8); CD10, CALLA, present on germinal-centre B-lineage cells but not other tonsil B cells (AL2; ref. 27); CD38, identifies germinal-centre B-lineage cells and plasma cells, but not other B cells found in the tonsil (OKT10; ref. 28); CD39, an antigen present on most tonsillar B cells, other than germinal-centre B-lineage cells, as well as some macrophages and plasma cells (AC-2; ref. 8); CD3, present on all T cells (OKT3; ref. 29); anti-IgG (8A4; ref. 30); anti-IgM (AF6; ref. 8). Sheep antibodies against human: IgA, IgD and IgM and IgG were used (The Binding Site, Birmingham, UK). Abbreviations: +ve, positive; -ve, negative.

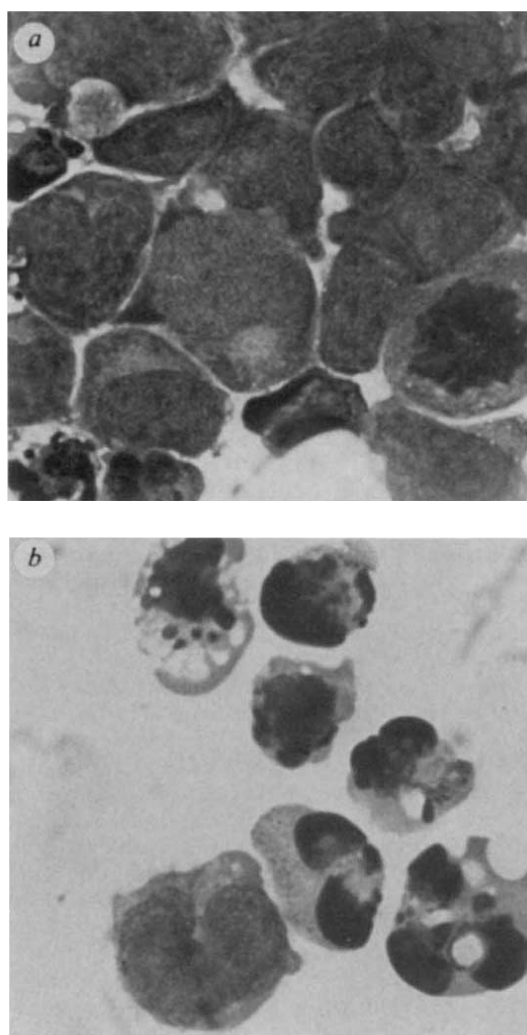


FIG. 1 a, Cells from the germinal-centre cell fraction of human tonsil after 6 h culture as described in Table 1. One intact centroblast is seen with six apoptotic cells which show the nuclear condensation and fragmentation which characterize this process⁶. This is a Romanowski-stained cytocentrifuge preparation photographed at a microscope magnification of $\times 1,000$. b, Cells from the same germinal-centre fraction depicted in a, cultured with sheep red cells coated with polyvalent anti-human immunoglobulin for 16 h as described in Table 1. Staining and photography as in a. Blast cells with frequent mitotic figures characterize these preparations. The cells tend to grow in clusters with some apoptotic cells still found at the periphery of these clusters.

TABLE 2 Selective cell death in germinal-centre B cells

Tonsil B-cell fraction	% cells recovered from cultures after:			
	16 h	40 h	72 h	96 h
High-density resting B cells	96-99	60-81	54-58	30-39
Low- and medium-density CD39 and sIgD -ve	22-26	0-0	0-0	0-0
Low- and medium-density CD39 and/or sIgD +ve	64-73	48-56	41-50	20-30
Low- and medium-density CD38 +ve	38-42	19-21	10-12	2-8
Low- and medium-density CD38 -ve	87-95	68-90	68-90	50-85

The first three tonsil B-cell fractions were prepared as described in Table 1, the CD38 positive (+ve) and CD38 negative (-ve) fractions were prepared in a similar way using CD38-coated sheep red cells. The CD38 +ve fraction contains all germinal-centre B-lineage cells but does include some cells from outside germinal centres (Table 1). Cell fractions were cultured for the periods indicated in flat-bottomed microtitre wells at 37 °C in a moist atmosphere of 5% CO₂ in air in 200 µl of RPMI 1640 medium enriched with 10% fetal bovine serum. The starting cell concentration was 2 × 10⁵ cells per ml. The number of viable cells recovered in two separate experiments from wells after the periods of culture indicated is shown. Cell viability was assessed by trypan-blue exclusion.

cytolysis^{12,13}. The macrophage content of the germinal-centre cell fraction (Table 1) is similar to that in the fraction positive for CD39 or IgD, or both. It is difficult to understand why there should be selective macrophage-mediated lysis of the germinal-centre cells and why this should be inhibited by CD40. If the germinal-centre cells are not being killed, they must be killing themselves. The high physiological cell death rate among these cells *in vivo*^{14,15}, together with the finding that other tonsil B cell populations do not destroy themselves by apoptosis on culture (Table 2), seems to argue against this being an artefact of the culture system.

Affinity maturation of antibodies in responses to T-cell-dependent antigens is mainly attributable to alteration of the structure of the immunoglobulin variable region by somatic mutation⁴. The time of onset of this somatic mutation¹⁶ correlates with the start of the germinal-centre reaction^{15,17}. Other features consistent with a link between these processes have been described previously⁵. Mutations can increase, decrease or fail to modify the affinity of antibodies. The experiments reported here indicate how centrocytes with low affinity for antigen can be eliminated. The germinal-centre cells with high affinity are most likely to be those which survive to give rise to memory B cells^{18,19}.

TABLE 3 Ability of antibodies to prevent cell death

a, Soluble CD40 antibody and anti-human immunoglobulin				
	% Viable cells (mean ± s.e.m. of 5 expts) recovered after:			
Culture additive	16 h	40 h	72 h	96 h
None	20 ± 6	0	0	0
Anti-Ig-coated sheep red cells	76 ± 6	23 ± 4	6 ± 8	0
Soluble CD40*	75 ± 12	39 ± 16	17 ± 4	0
Anti-Ig-coated sheep red cells plus soluble CD40*	86 ± 6	70 ± 12	40 ± 18	40 ± 24
b, Antibodies against different human immunoglobulin classes				
Antibody added to coat sheep red blood cells	% Viable cells (mean ± s.e.m. of 5 expts) recovered after 16-h culture			
None	20 ± 10			
Polyvalent anti-Ig	70 ± 8			
anti-IgG	54 ± 8			
anti-IgM	24 ± 10			
anti-IgD	26 ± 8			
anti-IgA	23 ± 3			

a, The prevention of cell death in cultures of germinal-centre cells by the addition of soluble CD40 antibody and sheep red cells coated with anti-human immunoglobulin. b, The relative ability of antibodies against different human immunoglobulin classes to prevent cell death in isolated germinal-centre cells. The germinal-centre cells used in these experiments were isolated as described in Table 1. They were cultured and their viability assessed as described in Table 2. The anti-immunoglobulins (Ig) used and the method used for their conjugation to sheep red cells is specified in Table 1.

* CD40 antibody G28-5 was added to cultures at a final concentration of 5 µg ml⁻¹ (ref. 7).

Defects in the selection system identified in this study could be involved in the pathogenesis of low-grade follicular lymphomas. These lymphomas have a (14; 18) translocation involving apposition of the proto-oncogene *bcl-2* and the immunoglobulin heavy-chain locus^{20,21}. Vaux *et al.*²² showed that expression of *bcl-2* transfected into IL-3-dependent cell lines prevented cell death on withdrawal of IL-3. The introduction of constructs representing the *bcl-2*-immunoglobulin gene fusion into the germ line of mice leads to the accumulation of mature B cells in the adult mice²³. It is an intriguing possibility that the *bcl-2* gene product may be involved in overriding the apoptotic tendency of germinal-centre cells. □

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Blockage of $\alpha\beta$ T-cell development by TCR $\gamma\delta$ transgenes

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T LYMPHOCYTES recognize antigens by means of T-cell receptors (TCR) composed of $\alpha\beta$ (refs 1-3) or $\gamma\delta$ heterodimers⁴⁻⁶. The mechanism governing the development of $\alpha\beta$ - and $\gamma\delta$ -bearing T cells from a common precursor T cell is so far unknown. It has been proposed that T-cell precursors rearrange their γ - and δ -chain genes first, and $\alpha\beta$ T cells are generated only from those cells that fail to rearrange productively both γ - and δ -chain genes^{7,8}. Our recent study on $\gamma\delta$ -transgenic mice contradicted this

hypothesis, however, and indicated that repression of γ -chain gene expression mediated by a transcriptional silencer element has a critical role in the generation of $\alpha\beta$ T cells⁹. Here we report that the generation of $\alpha\beta$ T cells is severely blocked in transgenic mice carrying γ - and δ -chain transgenes without the associated silencer, thereby strengthening the validity of the silencer model of T-cell development.

Our recent analysis of $\gamma\delta$ -transgenic mice (called KN6 transgenic mice) constructed with relatively long (~40 kilobases (kb) each) $V_4J_1C_1\gamma$ and $V_5DJ_1C\delta$ genomic DNA clones indicated that, in contrast to the Pardoll-Allison model^{7,8}, introduction of productively rearranged γ - and δ -chain genes into the germline does not prevent the rearrangement of the endogenous α - and β -chain genes and their cell-surface expression⁹. In these transgenic mice, transcription of the γ -chain transgene is repressed in $\alpha\beta$ T cells apparently through a *cis*-acting DNA element (silencer) present in the flanking region(s) of the γ -chain gene. This led us to propose a model of T-cell development in which commitment of a given T-cell precursor to the $\alpha\beta$ -lineage depends on the activation of the machinery acting on the silencer⁹. We have now constructed transgenic mice with γ - and δ -chain genes carrying no silencer element so as to perform the crucial test of the silencer model. The model predicts that the transgenes will be transcribed in all T cells in these mice, as opposed to the KN6 transgenic mice, and that their subsequent surface expression will prevent rearrangement and expression of TCR $\alpha\beta$ -chain genes, in analogy to the immunoglobulin system¹⁰.

As transgenes we used 15-kb and 21-kb genomic DNA fragments (Fig. 1) containing productively rearranged $V_5J_1C_1\gamma$ and $V_1D_2J_2C\delta$ genes, respectively, known to encode the $\gamma\delta$ TCR expressed on the surface of most early fetal thymocytes^{11,12} and dendritic epidermal $\gamma\delta$ T cells (DEC)^{11,13}. According to our previous study it is probable that the 15-kb γ -chain gene clone lacks the putative silencer element, because the transcription of a γ -chain transgene containing similar flanking regions is not repressed in $\alpha\beta$ T cells⁹. We analysed the progeny of two transgenic founders (henceforth called DEC transgenic mice).

Cell-surface expression of transgene-encoded TCR was investigated by flow cytometry, using monoclonal antibodies reactive with all $\gamma\delta$ TCR (3A10)¹⁴ or $V_{\gamma 5}$ -encoded γ -chains only (F536)¹¹. Consistent with previous reports, few if any $\gamma\delta$ T cells from nontransgenic adult mice expressed $V_{\gamma 5}$ -encoded $\gamma\delta$ TCR on their surface (Fig. 2a)^{11,12}. By contrast, more than 95% of

$\gamma\delta$ thymocytes or splenic T cells from the DEC transgenic mice were reactive with the F536 monoclonal antibody (Fig. 2a, and data not shown), indicating that, like the KN6 $\gamma\delta$ transgenic mice (Fig. 2a and ref. 9), the transgenes dominated the $\gamma\delta$ T cells for the expression of their TCR. The proportion of $\gamma\delta$ T cells in the thymus from DEC transgenic mice (25–75%) was dramatically increased in comparison with nontransgenic mice (0.5%) or KN6 transgenic mice (4.5%) (Fig. 2a). In addition, unlike KN6 transgenic mice, the thymus size was severely reduced in DEC transgenic mice (data not shown), suggesting that the generation of the main thymocyte subpopulation, that is, $\alpha\beta$ TCR⁺ cells, was strongly hampered in these animals.

This supposition was confirmed by immunofluorescence analysis using the anti- $\alpha\beta$ TCR monoclonal antibody H57-597¹⁵; less than 3% of thymocytes from 7-day-old DEC transgenic mice were $\alpha\beta$ TCR⁺, whereas most (>60%) thymocytes from the age-matched control mice or KN6 transgenic mice carried $\alpha\beta$ TCR (Fig. 2b). The proportion of $\alpha\beta$ TCR⁺ thymocytes increased with age to reach 50% in 10-week-old mice (Fig. 2b). But because the total number of thymocytes was greatly reduced in the DEC transgenic mice, the absolute number of $\alpha\beta$ chain-bearing thymocytes never reached more than 5% of the level observed in nontransgenic mice (data not shown).

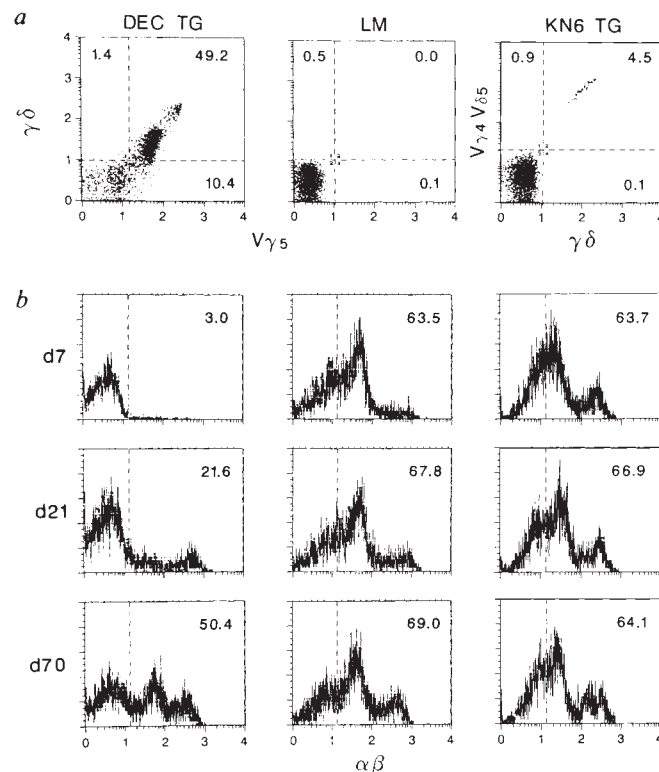


FIG. 2 Surface expression of $\gamma\delta$ and $\alpha\beta$ TCR by thymocytes from DEC transgenic, KN6 transgenic and nontransgenic mice. **a**, Thymocytes from 70-day-old DEC transgenic (left) and nontransgenic (middle) litter-mate mice were stained with anti- $V_{\gamma 5}$ monoclonal antibody F536 followed by fluorescein isothiocyanate (FITC)-coupled goat anti-hamster IgG and with biotinylated anti- $\gamma\delta$ -chain monoclonal antibody 3A10 followed by phycoerythrin-streptavidin. Shown are the dot-plot histograms (F536, horizontal axis; 3A10, vertical axis) obtained after flow cytometric analysis using a FACSCAN (Becton-Dickinson) and the percentage of cells in each quadrant. Fluorescence intensity is expressed in $\log_{10}$ units. For comparison is shown the dot-plot histogram (3A10, horizontal axis; 8D6 (anti- $V_{\gamma 4}V_{\delta 5}$)¹⁴, vertical axis) of thymocytes from age-matched KN6 transgenic mice⁹ (right). **b**, Thymocytes from 7-, 21- and 70-day-old DEC transgenic (left), nontransgenic (centre) and KN6 transgenic (right) mice were stained with biotinylated anti- $\alpha\beta$ -chain monoclonal antibody H57-597 followed by phycoerythrin-streptavidin. The percentages of positive cells are indicated to the right of each histogram. Fluorescence intensity is expressed in $\log_{10}$ units.

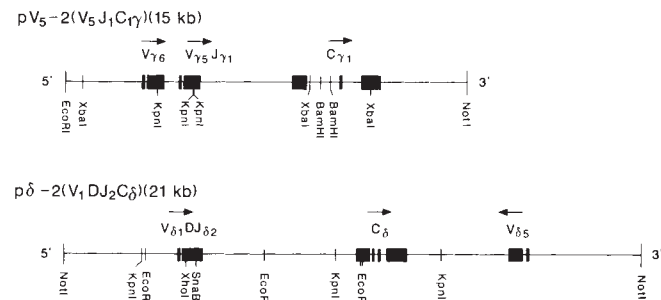


FIG. 1 Restriction maps of γ - and δ -chain genomic DNA clones used for the construction of transgenic mice. Filled boxes designate exons or gene segments; arrows above indicate transcriptional orientation.

METHODS. High-relative molecular mass DNA was isolated from a $\gamma\delta$ T-cell hybridoma KI-129¹² as previously described²² and was used to construct a cosmid library using the plasmid pWe15 vector²³. The library was screened with ³²P-labelled $V_{\gamma 5}$, C_{γ} , $V_{\delta 1}$ or C_{δ} probes for the cloning of the γ - and δ -chain genes. The C_{γ} and C_{δ} probes have been previously described^{24,25}. Screening led to the isolation of clones pV5-2 and p δ -2, containing the entire γ - and δ -chain genes expressed by the KI-129 hybridoma. The junctional sequences of clones pV5-2 and p δ -2 were identical to those published^{13,26}. The $V_{\gamma 5}$ gene was assigned according to ref. 27. The $\gamma\delta$ -transgenic mice were generated after injection into fertilized mouse eggs of purified pV5-2 and p δ -2 DNA as previously described^{5,28}.

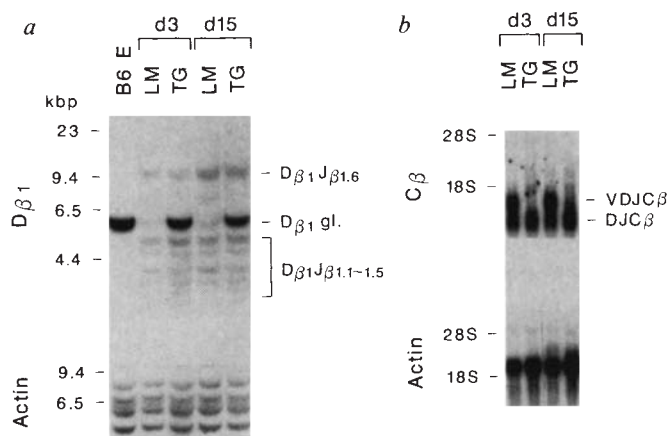


FIG. 3 Southern and northern blot analysis of DNA and RNA, respectively, from total thymocytes of DEC transgenic mice and nontransgenic littermates. *a*, Genomic DNA of thymocytes from 3- and 15-day-old DEC transgenic (TG) and nontransgenic (LM) mice and from C57BL/6 embryo (B6 e) were digested with *PvuII*, separated and transferred to nitrocellulose membrane. The Southern blot was hybridized with $D_{\beta 1}$ or actin probes. gl. Germ line DNA. DJ_{β} 1.1–1.6 bands were assigned according to ref. 29. *b*, RNA of thymocytes from 3- and 15-day-old DEC transgenic (TG) and nontransgenic (LM) mice was electrophoresed, transferred onto nitrocellulose membrane and hybridized to C_{β} or actin probes. The position of the 18S and 28S ribosomal RNA and that of $VDJ C_{\beta}$ and $DJ C_{\beta}$ transcripts are indicated. METHODS. Genomic DNA from thymocytes was purified as previously described²², digested with *PvuII* restriction endonuclease, separated (5 μ g lane⁻¹) on a 0.6% agarose gel and transferred to GeneScreen Plus membrane (NEN) according to ref. 30. Hybridization with ³²P-labelled $D_{\beta 1}$ probe (*PstI* fragment (1.06 kb) from the clone encoding germ-line $D_{\beta 1}$ DNA³¹) and actin (*PstI* fragment (0.8 kb) from a complementary DNA clone³²) probes was carried out as previously described³⁰. DNA probes were labelled by a random priming method³³. The autoradiographic bands were detected and intensities quantitated using a FUJI Bioimage Analyzer BA100³⁴. Total RNA was extracted using the guanidium thiocyanate–CsCl gradient method³⁵ and electrophoresed (10 μ g lane⁻¹) through formaldehyde containing 1.2% agarose gel²². The RNA was transferred to GeneScreen Plus membrane and baked at 80 °C for 2 h. Northern blot hybridization with ³²P-labelled probes was performed under the same conditions as that of the Southern blot.

The above results indicate that introduction into the germ line of productively rearranged 'short', in contrast to 'long', γ - and δ -chain transgenes severely hampers the development of $\alpha\beta$ T cells. To further investigate the mechanism by which $\alpha\beta$ T-cell differentiation is blocked, we analysed the DNA from thymocytes of young (3- and 15-day-old) DEC transgenic mice by the Southern blot method using a $D_{\beta 1}$ probe (Fig. 3*a*). In nontransgenic thymocytes, the germ-line $D_{\beta 1}$ band was barely detectable, reflecting the fact that incomplete *DJ* or complete *VDJ* rearrangements had taken place in most cells. By contrast, most $D_{\beta 1}$ gene segments remained in the germ-line configuration and the rest in the *DJ* state in the young transgenic mice (Fig. 3*a*). Consistent with the results of the Southern blot analysis, only low levels of full-size TCR β -chain messenger RNA (1.6 kb) were detected in transgenic thymocytes in comparison with nontransgenic thymocytes (Fig. 3*b*). Because *VJ α* rearrangements were only partly inhibited in DEC transgenic mice (data not shown), these results strongly indicate that the primary mechanism by which the DEC $\gamma\delta$ transgenes prevent $\alpha\beta$ T-cell development is the blockage of complete *VDJ β* gene rearrangements. If this is indeed the case, why then do $\alpha\beta$ T cells develop in older DEC transgenic mice? Northern blot analysis of $\alpha\beta$ and $\gamma\delta$ T-cell hybridomas from DEC transgenic mice shows that the levels of γ - and δ -chain transgene transcripts are lower in the former than in the latter (data not shown), suggesting that the level of $\gamma\delta$ -TCR transcripts must be above a certain threshold to efficiently inhibit rearrangements of endogenous TCR genes. The $\alpha\beta$ T cells observed in older DEC transgenic mice, then, are probably due to the accumulation of rare T cells in which the level of the transgene expression was below this threshold.

The drastic reduction in the number and the retardation of the appearance of $\alpha\beta$ T cells in the DEC transgenic mice is perfectly consistent with the silencer model of T-cell development. The possibility must be considered, however, that $\alpha\beta$ T cells are actually produced in these mice but are deleted in the thymus because of their coexpression of the transgene-encoded $\gamma\delta$ TCR. Although nothing is known about the ligand specificity of the transgene ($V_5J_1C_1\gamma$ and $V_1D_2J_2C\delta$)-encoded TCR, it is conceivable that its expression in the $\alpha\beta$ -cell lineage leads to negative selection of $\alpha\beta$ T cells. To investigate this possibility, we crossed the DEC transgenic mice with $\alpha\beta$ -transgenic mice¹⁶. If the silencer model is correct, then the introduction of functional α - and β -chain genes into DEC $\gamma\delta$ -transgenic mice would be expected to restore $\alpha\beta$ T cells. As shown in Fig. 4, ~80% of thymocytes from quadruple $\alpha\beta\gamma\delta$ -transgenic mice expressed $\alpha\beta$

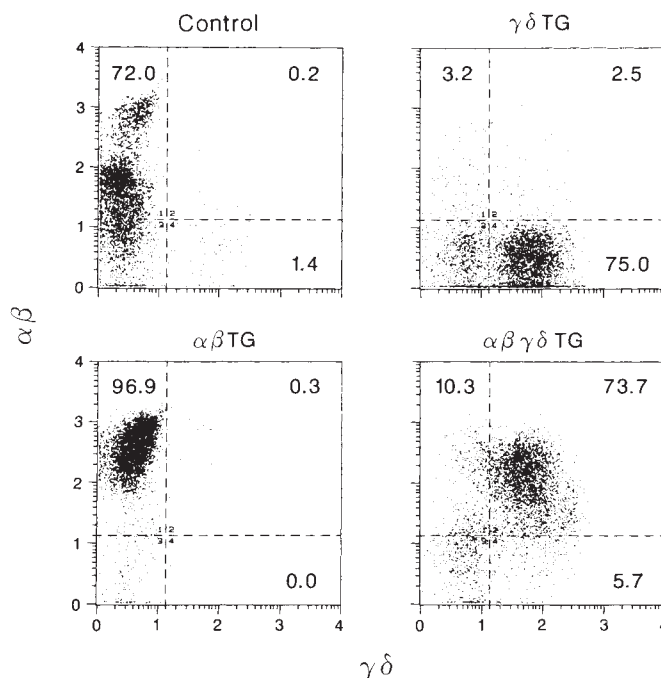


FIG. 4 Surface expression of $\gamma\delta$ and $\alpha\beta$ TCR by thymocytes from female $\gamma\delta$ -, $\alpha\beta$ - and $\alpha\beta\gamma\delta$ -transgenic mice. Thymocytes from 3-day-old DEC transgenic ($\gamma\delta$ -TG), $\alpha\beta$ -transgenic ($\alpha\beta$ -TG), $\alpha\beta\gamma\delta$ -transgenic ($\alpha\beta\gamma\delta$ -TG) and nontransgenic (LM) mice were stained with FITC-coupled anti- $\gamma\delta$ -chain monoclonal antibody 3A10 and biotinylated anti- $\alpha\beta$ -chain monoclonal antibody H57-597 followed by phycoerythrin–streptavidin. The dot-plot histograms (3A10, horizontal axis; H57-597; vertical axis) obtained after cytofluorometric analysis are shown as well as the percentage of cells in each quadrant. Fluorescence intensity is expressed in $\log_{10}$ units.

METHODS. DEC transgenic females ($H-2^b$) were mated with $\alpha\beta$ -transgenic male mice¹⁶ ($H-2^b$) and their female progeny analysed 3 days after birth. Transgene expression was screened by staining thymocytes with anti- $V_{\beta 8}$ monoclonal antibody F23.1³⁶ and anti- $\alpha\beta$ monoclonal antibody H57-597, or with anti- $V_{\gamma 5}$ monoclonal antibody F536 and anti- $\gamma\delta$ monoclonal antibody 3A10. More than 95% of $\alpha\beta$ T cells from $\alpha\beta$ - and $\alpha\beta\gamma\delta$ -transgenic mice were $V_{\beta 8}^+$ and more than 98% of $\gamma\delta$ T cells from $\gamma\delta$ - and $\alpha\beta\gamma\delta$ -transgenic mice were $V_{\gamma 5}^+$.

TCR, whereas such cells were barely detectable in the thymus from DEC transgenic littermates. Furthermore, total numbers of $\alpha\beta$ thymocytes in $\alpha\beta$ - and $\alpha\beta\gamma\delta$ -transgenic mice were identical. Therefore we conclude that the drastic reduction in the number of $\alpha\beta$ T cells in the DEC transgenic mice is not due to intrathymic deletion of immature $\alpha\beta$ T cells.

Taken together, these observations strongly support the hypothesis that T-cell precursors are committed to generating $\alpha\beta$ T cells, independently of the rearrangement status of their γ -chain genes, and that this commitment involves activation of factor(s) that repress γ -chain gene transcription through interaction with a *cis*-regulatory element. Recently, Winoto and Baltimore¹⁷ found that most extrachromosomal circular DNA generated by V_{α} - J_{α} joinings retain the δ -chain gene segments in the germ-line configuration and proposed that the control of δ -chain gene rearrangement plays a key part in the separation of $\alpha\beta$ and $\gamma\delta$ T-cell lineages. But, Takeshita *et al.*¹⁸ reported that some ' α -circles' do contain rearranged δ -chain gene segments, suggesting that the control at the level of δ -chain gene rearrangement is insufficient.

It has previously been shown that, in contrast to C_1 -associated γ -chain genes, $V_2J_2C_2\gamma$ genes are transcribed in $\alpha\beta$ T-cell clones and hybridomas^{4,19-21}. The protein products of these genes have not been detected on the surface of freshly isolated T cells, however. Thus, the silencer model seems to apply to the separation of most, if not all, $\alpha\beta$ and $\gamma\delta$ T-cell lineages. □

A protein binding to the J_{κ} recombination sequence of immunoglobulin genes contains a sequence related to the integrase motif

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SITE-SPECIFIC recombination requires conserved DNA sequences specific to each system, and system-specific proteins that recognize specific DNA sequences. The site-specific recombinases seem to fall into at least two families, based on their protein structure and chemistry of strand breakage (reviewed in ref. 1). One of these is the resolvase-invertase family, members of which seem to form a serine-phosphate linkage with DNA. Members of the other family, called the integrase family, contain a conserved tyrosine residue that forms a covalent linkage with the 3'-phosphate of DNA at the site of recombination. Structural comparison of integrases shows that these proteins share a highly conserved 40-residue motif². V -(D)- J recombination of the immunoglobulin gene requires conserved recombination signal sequences (RS) of a heptamer CACTGTG and a T-rich nonamer GGTTTTTGT, which are separated by a spacer sequence of either 12 or 23 bases (reviewed in refs 3 and 4). We have recently purified, almost to homogeneity, a protein that specifically binds to the immunoglobulin J_{κ} RS containing the 23-base-pair spacer sequence⁵. By synthesizing probes on the basis of partial amino-acid sequences of the purified protein, we have now isolated and characterized the complementary DNA of this protein. The amino-acid sequence deduced from the cDNA sequence reveals that the J_{κ} RS-binding protein has a sequence similar to the 40-residue motif of integrases of phages, bacteria and yeast, indicating that this protein could be involved in V -(D)- J recombination as a recombinase.

The J_{κ} RS-binding protein was purified almost to homogeneity from the murine pre-B-cell line 38B9 (Fig. 1a) by the procedure of Hamaguchi *et al.*⁵. The J_{κ} RS-binding protein with a relative molecular mass (M_r) of 60,000 (60K) was expressed in lymphoid cell lines but not in nonlymphoid cell lines. The protein binds to the J_{κ} RS with a dissociation constant (K_d) of 1 nM. DNase-I footprinting analysis and the binding kinetic assay with several mutant probes strongly indicate that the protein directly interacts with the heptamer sequence and that a mutation in the heptamer greatly reduces the affinity of the J_{κ} RS-binding protein for DNA. Our initial attempt to determine the N-terminal amino-acid sequence was unsuccessful, probably because of modification of the N-terminal residue. The purified preparation was then digested with trypsin, and the resulting peptides were fractionated by reverse-phase HPLC (Fig. 1b). Twenty-three peptides were re-chromatographed and their amino-acid sequences were determined. Two oligodeoxyribonucleotide probes were synthesized on the basis of partial amino-acid sequences of the tryptic peptide 21 and used to screen a cDNA library derived from 38B9 poly(A)⁺RNA (Fig. 2a, b). The probes hybridized with 2 of 1×10^6 colonies of the cDNA library. The two cDNA clones were purified and isolated by repeated screening. The clones designated as RBP-1 and RBP-2 contained 3.3- and 4-kilobase (kb) inserts, respectively. Restriction-site map-

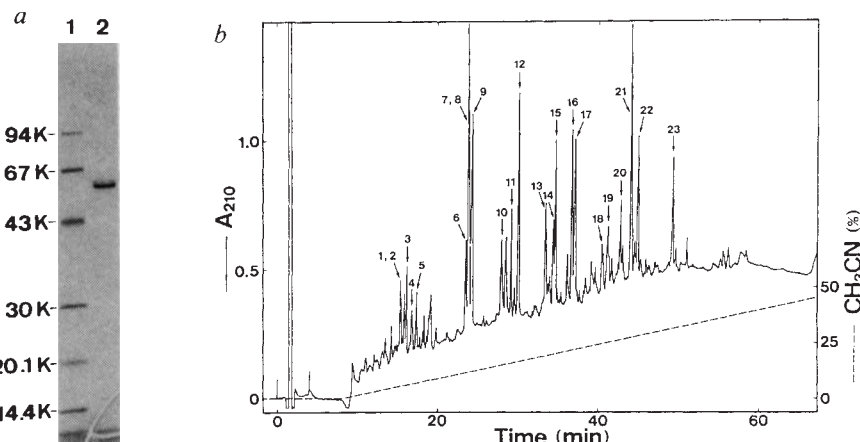
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FIG. 1 Purification and partial amino-acid sequence analysis of J_{κ} RS-binding protein. **a**, Coomassie brilliant-blue stain of an SDS-polyacrylamide gel. Lane 1, mixtures of size markers; lane 2, purified J_{κ} RS-binding protein (0.5 μ g). **b**, Reverse-phase HPLC of peptides formed by tryptic digestion of the purified J_{κ} RS-binding protein. Solid line and left-hand axis, absorbance at 210 nm (A_{210}); broken line and right-hand axis, acetonitrile concentration. Peptides corresponding to peaks 1–23 were separated by re-chromatography and sequenced. All the amino-acid sequences thus determined agreed completely with regions of the amino-acid sequence deduced from the cDNA sequence (see Fig. 2c).

METHODS. A murine pre-B cell line 38B9 (ref. 13) which rearranges immunoglobulin genes during *in vitro* culture was maintained in RPMI 1640 medium with 10% (v/v) heat-inactivated FCS. The detailed protocol for the purification of the J_{κ} RS-binding protein is described in ref. 5. In brief, the crude nuclear extract from 38B9 (8×10^{10} cells) was fractionated by heparin-agarose column chromatography, followed by J_{κ} RS probe-coupled agarose column chromatography. Aliquots of fractions collected were assayed for binding activity to the J_{κ} RS probe in a gel-retardation assay. Each column chromatography was repeated twice. The method of SDS-PAGE was as in ref. 5. The dialysed preparation (200 pmol) of affinity-purified J_{κ} RS-binding protein were



digested with trypsin. The digested peptides were fractionated by reverse-phase HPLC (Chemcosorb 30DS-H) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Each fraction was further separated by re-chromatography (μ -Bondaspher C-18 · 300A) to give a single absorbance peak. The amino-acid sequence of each peptide was determined using a gas-phase protein sequencer (Applied Biosystems).

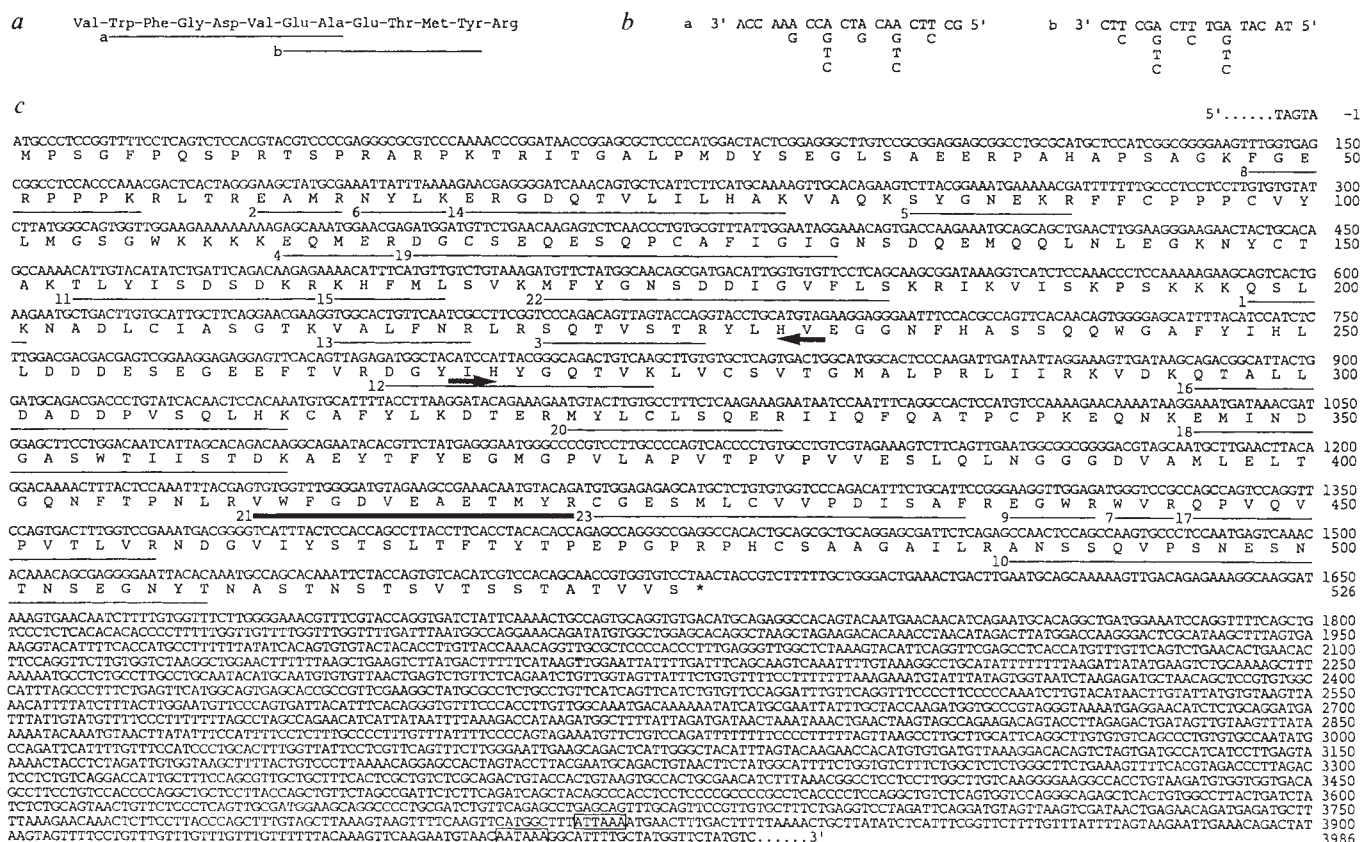


FIG. 2 Nucleotide sequence and corresponding amino-acid sequence of J_{κ} RS-binding protein. **a**, Amino-acid sequence of tryptic fragment 21. Sequences used for construction of probes **a** and **b** are indicated. **b**, Synthetic oligodeoxyribonucleotide mixtures used as probes **a** and **b**. **c**, Nucleotide sequence and predicted amino-acid sequence (single-letter code) of J_{κ} RS-binding protein. Thick and thin underlines indicate peptide 21 and other peptides sequenced from the purified protein, respectively. Poly(A)-addition signal sequences are boxed. Asterisk indicates termination codon. The boundaries of the integrase motif region (Fig. 4) are indicated by two horizontal arrows.

METHODS. A cDNA library linked to the SV40 early promoter was constructed from poly(A)⁺RNA of 38B9 cells, as described, by Okayama and Berg¹⁴. *E. coli* AG-1 transformants were screened with ³²P-labelled oligodeoxynucleotide probes **a** and **b** by the method of Hanahan and Meselson¹⁵. Nucleotide sequences were determined by the dideoxy method using plasmid pUC vector¹⁶. Oligonucleotides were synthesized by an automated synthesizer (Applied Biosystems).

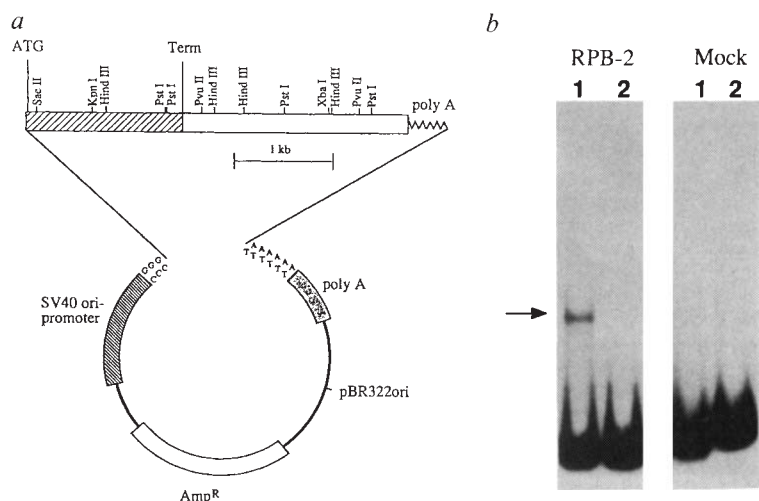
ping and nucleotide sequence determination showed that the two clones are identical, except that RBP-2 has an extra 550-base-pair (bp) sequence at the 5' end and uses the 140-bp downstream poly(A) addition site.

Nucleotide-sequence determination of the cDNA revealed an open reading frame that encodes 526 amino-acid residues (Fig. 2c). All the 23 partial amino-acid sequences determined (Fig. 1b) were identified in this amino-acid sequence deduced

from the cDNA sequence. The calculated M_r of the J_κ RS-binding protein as 57K (including the initiating methionine), in reasonable agreement with the value of 60K estimated by SDS-PAGE. Of the total 4,000-bp insert, the 3'-noncoding region occupies 2,400 nucleotides excluding the poly(dA) tract; two polyadenylation signal sequences are located 30 and 170 nucleotides upstream of the poly(dA) tract of RBP-2. The size of the J_κ RS-binding protein messenger RNA (4.5 kb) of 38B9 cells,

FIG. 3 a, Restriction map and structure of RBP-2 cDNA clone. The original RBP-2 clone in Okayama-Berg expression vector was used. Solid and zig-zag lines indicate vector and poly(A) tails, respectively. Black and open rectangles indicate coding and untranslated regions, respectively. b, Expression of RBP-2 cDNA in COS-7 cells. Nuclear extracts from RBP-2 and mock-transfected COS-7 cells were purified in a DNA-affinity column⁵, and were assayed for binding to the J_κ RS probe (lane 1) or its single-base substitution mutant probe, J_κ 1P3 (lane 2) in a gel-retardation assay⁵. Term, termination site.

METHODS. COS-7 (1.5×10^7 cells) maintained in DMEM medium with 5% FCS were transfected and cultured as previously described¹⁷. After 48 h of culture, RBP-2 and mock-transfected COS-7 cells were collected. Their nuclear extracts were prepared and dialysed against buffer A (50 mM NaCl, 20 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 1 mM DTT, 30% glycerol) as previously described⁵. Each nuclear extract was fractionated by affinity chromatography on a column containing 1 ml packed J_κ RS DNA-coupled agarose⁵. After sample application, the column was washed with five volumes of buffer A, and then eluted with buffer A containing 250 mM NaCl. For the gel-retardation assay, aliquots of eluted fractions containing the same amount of protein were incubated in reaction mixture (30 μ l total), containing 50 mM NaCl, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 30% glycerol with 2 ng radiolabelled



probe, 1 μ g poly(dI), poly(dC) for 30 min at 20 $^{\circ}$ C. The incubated sample was analysed by 4% PAGE as previously described⁵.

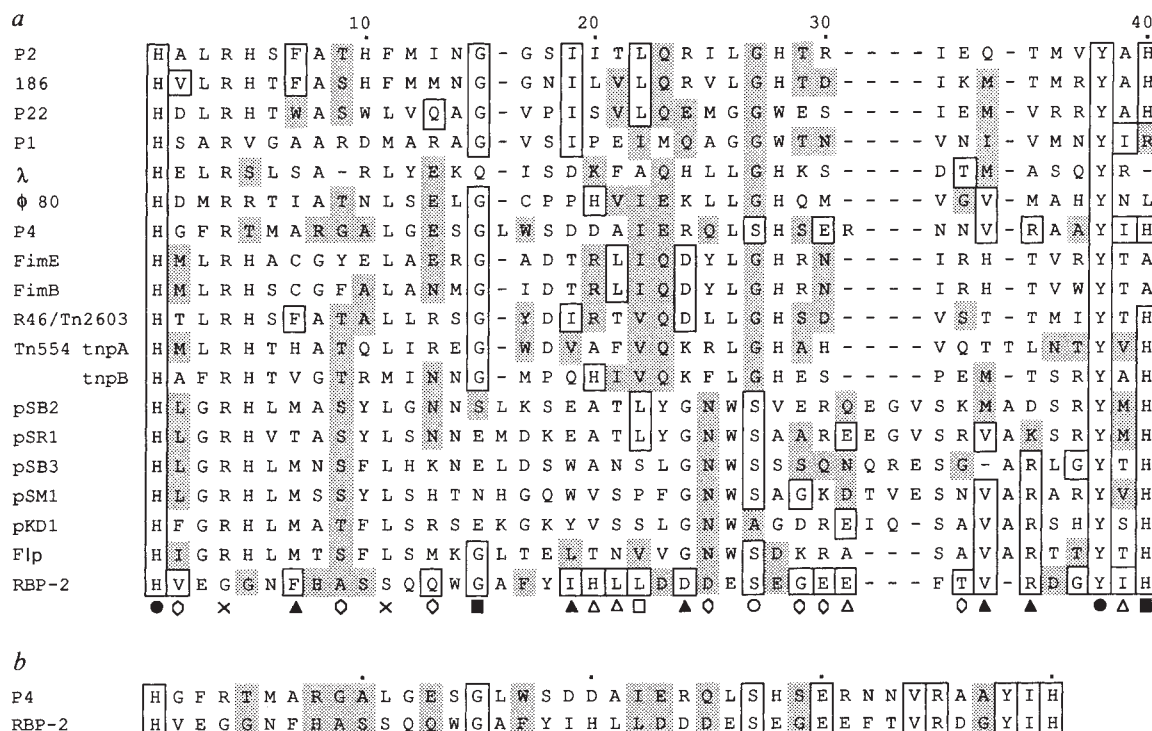


FIG. 4 Comparison of the 40-residue motif sequences of the J_κ RS-binding protein and recombinases of the integrase family (single-letter amino-acid code). a, Alignment with all the known sequences. b, Alignment with P4 integrase. Only residues that are conserved between the J_κ RS-binding protein (RBP-2) and any of the other proteins are boxed. Residues that belong to the same exchange groups with those of RBP-2 are shaded. Extents of conservations are indicated by closed and open symbols at the bottom, excluding and including, respectively, residues of the same exchange group. Circles, 100%; rectangles, >68%; triangles, >21%. Residues with

less than two identical members but more than seven members of identical exchange groups are indicated by open hexagons. Residues that are conserved >60% among the other recombinases, but not conserved in RBP-2, are shown by crosses. Origins of integrase sequences are: Phages P2 (ref. 2), 186 (ref. 2), P22 (ref. 2), P1 (ref. 2), λ (ref. 2), ϕ 80 (ref. 2), and P4 (ref. 18); FimE and FimB proteins (ref. 19); transposons Tn2603 (ref. 20) and Tn554 (ref. 21); plasmids pSB2 (ref. 22), pSR1 (ref. 23), pSB3 (ref. 24), pSM1 (ref. 22), and pKD1 (ref. 25); and Flp protein (ref. 26). Different reading frames for Tn554 indicated by tnpA and tnpB.

estimated by northern-blot hybridization analysis, is in general agreement with the size of the RBP-2 encoded protein if the additional poly(A) tract is considered (data not shown).

To confirm that the cDNA clones isolated encode the J_{κ} RS-binding protein, COS-7 cells were transfected with RBP-2 cDNA in an expression plasmid linked to the simian virus 40 (SV40) early promoter (Fig. 3a). After 48 h of culture, the nuclear extract of transfected COS-7 cells was prepared and fractionated by affinity chromatography using agarose coupled to J_{κ} RS DNA⁵. Aliquots of fractions bound to the affinity column were assayed for binding to the J_{κ} RS probe in a gel-regardation assay. Binding was detected in the extract of RBP-2-transfected COS-7 cells but not in mock-transfected COS-7 cells (Fig. 3b). Furthermore, a marked reduction in the binding affinity was observed when a mutant probe (J_{κ} 1P3 probe) with a single base substitution of the third G in the heptamer was used (Fig. 3b), consistent with our previous observation that the GTG sequence of the heptamer interacts with the J_{κ} RS-binding protein⁵. These results strongly indicate that the RBP-2 cDNA sequence encodes the J_{κ} RS-binding protein. This protein has a basic residue cluster (Lys-Lys-Lys-Lys for residues 107–110) which often serves as a nuclear-localization sequence⁶.

A computer survey did not reveal any other proteins that have extensive sequence homology with the overall sequence of the J_{κ} RS-binding protein. The protein does not seem to have any DNA-binding domains such as zinc-finger⁷, helix-turn-helix⁸, or leucine-zipper⁹ structures. But the J_{κ} RS-binding protein contains a sequence similar to the 40-residue motif of the integrase family that includes λ phase integrase, fimbriae switch recombinase of *Escherichia coli* and yeast Flp recombinase² (Fig. 4a). In the 40-residue motif, three residues (His 1, Arg 4 and Tyr 38) are perfectly conserved among all the 18 integrase-family proteins so far sequenced. The conserved tyrosine residue (Tyr 38), of Flp^{10,11} and λ phage integrase¹² is involved in the catalytic reaction of the integrase. The J_{κ} RS-binding protein conserves the His 1 and Tyr 38 residues, but not the Arg 4 residue. In addition, Leu 11 is >60% conserved among the other recombinases, but is not conserved in the J_{κ} RS-binding protein. Nonetheless, the J_{κ} RS-binding protein contains two highly (>68%) conserved residues (Gly 15 and His 40) and seven moderately (>21%) conserved residues within the 40-residue motif. If we regard residues in the same exchange group as conserved, 21 out of 40 residues of the motif region of the J_{κ} RS-binding protein are >32% conserved among all the 19 proteins.

Out of the other 18 recombinases, the J_{κ} RS-binding protein has the highest homology with the P4 integrase (Fig. 4b). The 40-residue motifs of these two proteins can be aligned without any deletions and insertions, and share 9 identical and 11 same-family residues. Furthermore, the first 16 residues of the recognition sequence (GAGTCCGGCCTTCGGCACCA) for the P4 integrase¹⁸ has some homology with the sequence of the heptamer-nonamer of the immunoglobulin gene, that is, GAGTC(A)CGGTTTTTGT.

In view of the large evolutionary distance between bacteriophages and mouse, such homology indicates that this motif in the J_{κ} RS-binding protein could be conserved to maintain the function of the protein as a recombinase. It is of interest that the primary structures of recombinases of the integrase family are divergent except for this homologous 40-residue motif, which seems to be a catalytic domain but not a binding domain for DNA. *In vitro* studies of site-specific recombination indicate that the reaction is essentially catalysed by a single protein that seems to be required in stoichiometric rather than catalytic amounts, although additional accessory proteins are required in some cases¹. Experiments are in progress to examine whether the J_{κ} RS-binding protein catalyses or stimulates V-(D)-J recombination, although other accessory binding proteins, such as integration host factor¹, could be required simultaneously for efficient recombination. □

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Genetic evidence that *ZFY* is not the testis-determining factor

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In mammals, the testis determining gene (*TDF*), present on the Y chromosome, induces the undifferentiated gonads to form testes¹. The position of *TDF* on the human Y chromosome has been defined by analysing the genomes of XX males and XY females², generated by abnormal genetic exchange between the X and Y chromosomes in male meiosis³. In this way *TDF* has been localized close to the pseudoautosomal region shared by the sex chromosomes, in the distal Y-specific region. A recently cloned human gene, *ZFY*, has many features indicating that it is *TDF*^{4–6}. For example, *ZFY* encodes a protein with many features of a transcription factor including a domain with multiple 'zinc-finger' motifs^{4–6}. Less consistent with *ZFY* being *TDF*, however, is the presence of a very similar gene, *ZFX*, on the X chromosome^{4,7}, and the presence of a sequence related to *ZFY* on autosomes in marsupials⁸. We now report on analysis of XX males lacking *ZFY*. In these individuals, the male phenotype could be explained by a mutation in a gene 'downstream' of *ZFY* in the sex-determining hierarchy; but in that case there should be no exchange of material between the X and Y chromosomes. We find on the contrary that in 4 XX males lacking *ZFY*, there is exchange of Y-specific sequences next to the pseudoautosomal boundary, redefining the region in which *TDF* must lie.

By studying the sequences present in an XX male (LGL203) and absent in an XY female (WHT1013), Page *et al.* had previously localized *TDF* to within 140 kilobases (kb) (ref. 4 and Fig. 1). The latter patient, however, was not a typical exchange XY female, but had a translocation between the Y chromosome and chromosome 22. The sequences between the breakpoints in these patients were isolated and the gene *ZFY* was identified in this region.

TABLE 1 Mapping of breakpoints in XX sex-reversed individuals

Patient	Origin	Karyotype	Hypospadia	Internal genitalia	Testis biopsy	HfO.2 (Y-boundary)	H2.1	P0.9	Probe E1.3	27A	GMGY3	pMF-1 (ZFY)
ZM	Algeria	46 XX	Anterior	Vaginal pouch	Leydig cells Sertoli cells No spermatogonia	+	+	+	—	—	—	—
MB	France	46 XX	None	Normal male†	Leydig cells Sertoli cells No spermatogonia	+	+	+	—	—	—	—
DL*	France	46 XX	Posterior	Uterus	Bilateral ovotestis	+	+	+	—	—	—	—
TL*	France	46 XX	Anterior	Normal male	Sertoli cells No spermatogonia	+	+	+	—	—	—	—

* Familial case.

† Patient presented with bilateral cryptorchidism.

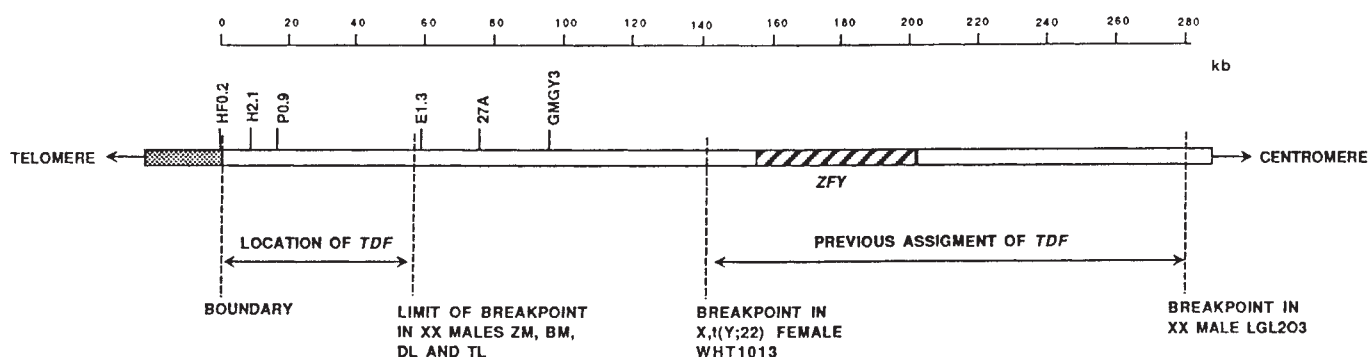


FIG. 1 Map of distal short arm of Y chromosome: stippled region at left is the pseudoautosomal region. Cross-hatched region shows the position of *ZFY* identified by Page *et al.*⁴. The breakpoints in the XX male and XY female used in the localization of *ZFY* are shown. Individual probes used to define the breakpoints in the XX males reported here were isolated from overlapping

clones obtained while walking along the Y chromosome⁹, except probes 27a and GMGY3, which were isolated previously^{15,16}. HfO.2 derives from the pseudoautosomal region and is present on both X and Y chromosomes. The clones H2.1, P0.9, E1.3, 27a and GMGY3 are specific for the Y chromosome.

In previous reports we have described the isolation and structure of the boundary between the pseudoautosomal region and the sex chromosome-specific regions⁹. If *ZFY* is *TDF*, it should be predicted that all exchange XX males carry *ZFY*, whereas all XX males lacking *ZFY* are due to mutations elsewhere in the genome. The latter class cannot be derived by terminal X-Y exchange and should consequently lack the Y chromosome-derived pseudoautosomal boundary. Using a polymerase chain reaction (PCR)-based assay, 14 *ZFY*-negative XX males were screened for the presence of the Y chromosome-derived pseudoautosomal boundary: three XX males and one XX intersex individual were found to have the Y pseudoautosomal boundary (Fig. 2). Repeating these experiments by Southern blotting confirmed that all four sex-reversed individuals were positive for both the X and Y boundaries and were *ZFY*-negative (Fig. 3). The relative signal intensities between the X and Y boundary fragments excludes mosaicism and chimaerism as explanations of the results. The possibility that the boundary position is polymorphic is also unlikely as the boundary structure and position is invariant in more than 150 individuals tested; moreover, the Y boundary is in the same position in the great apes (ref. 9 and unpublished observations). The position of the exchanges in the four exceptional XX sex-reversed individuals was mapped by testing for reaction with a series of probes located between the boundary and *ZFY* (Fig. 3). The results summarized in Table 1 and in Fig. 1 show that all the breakpoints are clustered between the sequences defined by probes P0.9 and E1.3. The region of the Y chromosome present in these sex-reversed individuals does not include the region defined by Page *et al.*⁴ as the sex-determining region. The simplest interpretation of these results is that *TDF* lies within 60 kb of the pseudoauto-

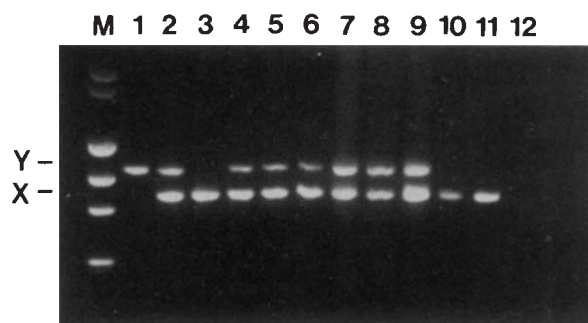
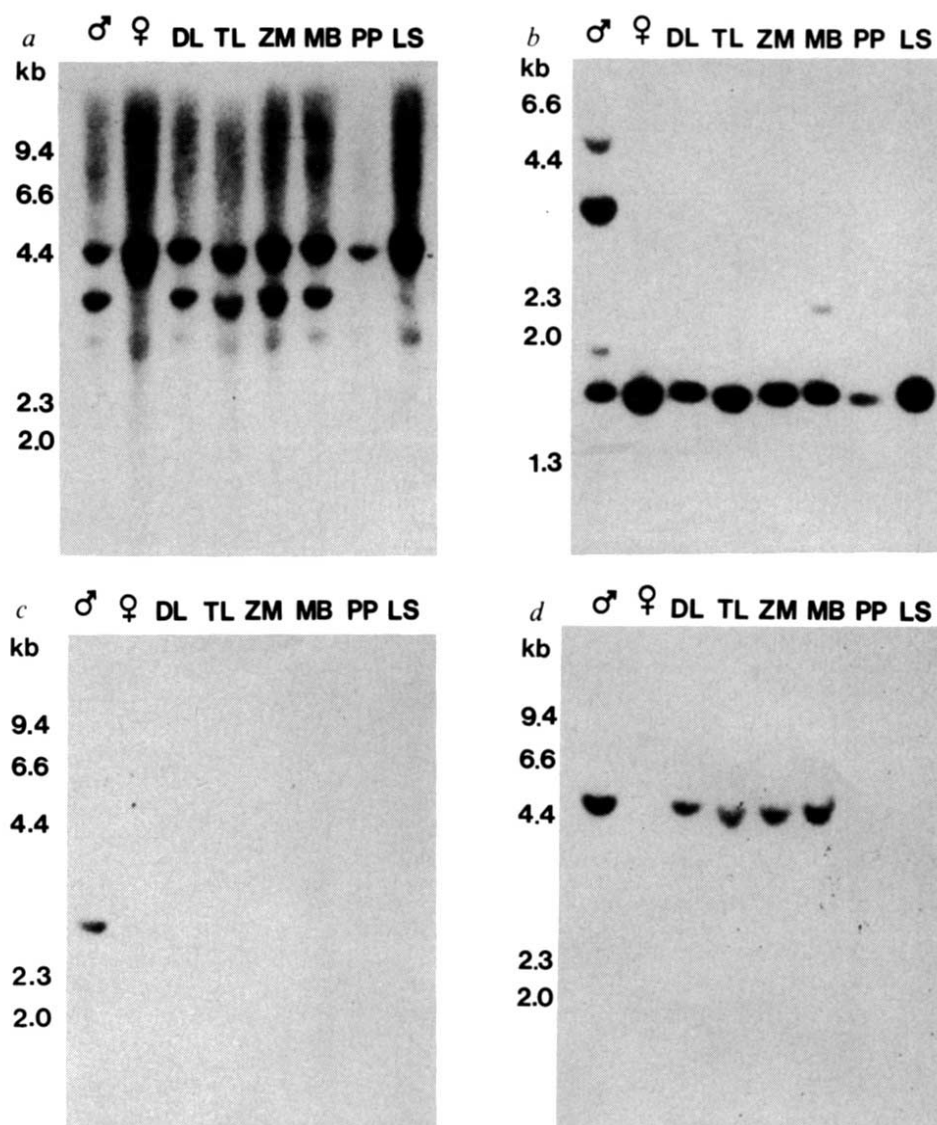


FIG. 2 PCR analysis of X and Y boundaries. Oligonucleotides specific for X- or Y-chromosomal sequences were used together with an oligonucleotide from the pseudoautosomal region as primers in PCR reactions¹⁷. The Y-specific fragment is 1.1 kb and the X-specific fragment, 0.95 kb. The size markers in lane M are 2.3- and 2.0-kb λ -HindIII fragments, and 1.35-, 1.0-, 0.87- and 0.60-kb ϕ X173 *Hae*III fragments. PCR was performed on 1 μ g genomic DNA from the following sources: (1) 3E7, a mouse-human hybrid containing the human Y chromosome as its sole contribution of human DNA¹⁸; (2) a normal XY male; (3) a normal XX female; (4) an XX male, SB, positive for *ZFY*; (5) an XX male J-JM positive for *ZFY*; (6) XX male, MB, negative for *ZFY*; (7) XX intersex, TL, negative for *ZFY*; (8) XX male, DL, brother of TL, negative for *ZFY*; (9) XX male, ZM, negative for *ZFY*; (10) XX male, PP, negative for *ZFY* and Y boundary; (11) XX male, LS, negative for *ZFY* and Y boundary; (12) no DNA added, control. Of fourteen *ZFY* XX males or intersex individuals analysed, four—MB, TL, DL, and ZM—were positive for the Y boundary, whereas the remainder—of which PP and LS are representative—were negative for the boundary. All XX males who had *ZFY* also had the Y boundary. Clinical details of the XX males will be published elsewhere¹⁹.

FIG. 3 Hybridization of probes to Southern blots. In each case filters contain the same lanes; ♂, normal male; ♀, normal female; DL, TL, ZM, MB, the four XX males discussed here, negative for *ZFY* but positive for the Y boundary; PP and LS, XX males negative for *ZFY* and the Y boundary. *a*, *SacI* digests probed with Hf0.2—a pseudoautosomal probe⁹. *SacI* gives a 4.5-kb X-specific fragment and a 3.2-kb Y-specific fragment across the pseudoautosomal boundary. *b*, *EcoRI* digests probed with a complementary DNA clone pMF-1, corresponding to a partial transcript of *ZFY* including the zinc-finger domain⁶. The three bands in the normal-male track not seen elsewhere are Y-specific. The weak 2.3-kb band seen in MB is probably due to a partial digestion and was not present in other digests of the same DNA sample in other experiments. *c*, *HindIII* digests probed with the Y-specific clone 27a¹⁵. Only the normal male was positive. *d*, *EcoRI* digests probed with the boundary proximal probe P0.9 (ref. 9). This probe is specific for the short arm of the Y chromosome.



somal boundary; this excludes *ZFY* as a candidate for *TDF*. These results also formally demonstrate that some XX males are not generated by terminal exchange and must be due to mutations elsewhere in the sex-determination pathway¹⁰.

The only discrepancy between our results and those in previous publications is the description by Page *et al.*⁴ of a female carrying a t(Y; 22) translocation which should include the Y pseudoautosomal boundary region. It is possible that this translocation is complex or, alternatively, *TDF* could have been inactivated by a position effect.

The four sex-reversed individuals vary in phenotype from normal male with testis, but lacking germ cells, to hermaphrodite with bilateral ovotestes and a uterus (Table 1). The variation in phenotype could be caused by the proximity of the breakpoints to *TDF*. Particularly striking are the two familial cases—the 'brother' and 'sister' TL and DL. Although the origin of these individuals is not clear, familial exchange XX males have been described previously¹¹, and one possibility is that the father has a mosaic gonad.

The results described here and by Koopman *et al.*¹² strongly argue against a role for *ZFY* in sex determination; *ZFY* could have a male specific function in spermatogenesis, however. Separate functions for *TDF* and *ZFY* have been proposed previously to explain the evolutionary relationship between the sex chromosomes of eutherians and metatherians¹³, and could be consistent with the autosomal location of *ZFY*-related sequences in marsupials⁸.

One explanation for the phenotypes we observe is that they result from the reciprocal breakpoints on the X chromosome. A more likely explanation is that the *TDF* gene lies close to, or even spans, the pseudoautosomal boundary. We are now testing these hypotheses. □

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Zfy gene expression patterns are not compatible with a primary role in mouse sex determination

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THE Y chromosome determines maleness in mammals. A Y chromosome-linked gene diverts the indifferent embryonic gonad from the default ovarian pathway in favour of testis differentiation, initiating male development^{1,2}. Study of this basic developmental switch requires the isolation of the testis-determining gene, termed *TDF* in humans and *Tdy* in mice. *ZFY*, a candidate gene for *TDF*, potentially encodes a zinc-finger protein³, and has two Y-linked homologues, *Zfy-1* and *Zfy-2*, in mice^{4,5}. Although *ZFY*, *Zfy-1* and *Zfy-2* seem to map to the sex-determining regions of the human and mouse Y chromosomes, there is no direct evidence that these genes are involved in testis determination. We report here that *Zfy-1* but not *Zfy-2* is expressed in differentiating embryonic mouse testes. Neither gene, however, is expressed in *W^e/W^e* mutant embryonic testes which lack germ cells. These observations exclude both *Zfy-1* and *Zfy-2* as candidates for the mouse testis-determining gene.

The primordial gonads (genital ridges) of male and female mouse embryos are indistinguishable until ~11.5 days post coitum (d.p.c.). By 12.5 d.p.c., testis differentiation is apparent from the presence of cords formed by the alignment of Sertoli cells. It is expected that the gene responsible for testis determination will be expressed around this stage of development. *Zfy-1* or *Zfy-2* expression is not detectable by northern blot analysis of RNA from whole mouse embryos⁶. To enrich for gonadal tissue, we isolated fetal genital ridges and gonads and analysed their RNA by northern blotting. *Zfy* transcripts were detected in adult testis but not in fetal gonads (Fig. 1a). By contrast, the X-linked homologue *Zfx* (ref. 5) was expressed at all stages. *Zfy* gene expression is clearly associated with germ cells in adult testes because transcripts were undetectable in XX *Sxr* and XX

Sxr' testes which lack germ cells^{7,8} (Fig. 1b).

Because of the relative insensitivity of northern blotting, we used the sensitive polymerase chain reaction (PCR) technique to look for *Zfy* transcripts in dissected embryonic tissues (Fig. 2). Whereas no transcripts were detected in 9.5 d.p.c. embryos, increasing levels of *Zfy-1* expression were seen between 10.5 and 12.5 d.p.c.; *Zfy-1* expression was maintained for at least two further days in the testes. Transcripts were absent from male nongonadal tissues. DNA blot analysis of the PCR products confirmed the onset of *Zfy-1* expression at 10.5 d.p.c. (Fig. 2a). The minor signal detected in the 12.5 d.p.c. ovary sample must reflect the occasional error in sexing embryos at this stage by gonadal morphology, given that RNA was derived from large numbers of pooled embryo samples. When RNA was prepared from genital ridges and gonads from individual embryos without pooling, a similar profile of *Zfy-1* expression between 10.5 and 14.5 d.p.c. was seen (Fig. 2b). In addition, decreasing levels of expression were found between 14.5 and 17.5 d.p.c.; all female samples were negative for *Zfy-1* expression.

By contrast, *Zfy-2* transcripts were detected in adult testes but not in embryonic mouse gonads at any of the stages examined (Fig. 2a). We conclude from this that *Zfy-2* is not *Tdy*.

The oligonucleotide primers used to detect *Zfy* transcripts were expected to give single PCR products of 477 base pairs (bp), based on published complementary DNA sequences^{6,9}. Instead, two additional products of ~520 and 570 bp were detected (Fig. 2b). Sequencing of DNA from each band from 12.5-d.p.c. testes revealed three alternatively spliced *Zfy-1* transcripts (Fig. 3). No stage-specific difference in their relative abundance was seen. There is no obvious significance to this alternative splicing, which is within the 5' untranslated portion of the messenger RNA. The additional exons in the longer *Zfy-1* transcripts do not introduce any alternative translational start sites associated with open reading frames, and would not alter the *Zfy-1* protein.

The embryonic gonad has both somatic and germ cell components, but it is clear that germ cells are not required for testis determination or differentiation^{10,11}. The *W* mutation in mice affects primordial germ cell proliferation and migration into the genital ridge¹²; in embryos homozygous for the most severe form, *W^e*, testes form normally despite being virtually devoid of germ cells (ref. 13; and M. Buehr, personal communication).

FIG. 1 Northern blot analysis of *Zfy* gene expression. **a**, Analysis of RNA from adult and embryonic mouse gonads and urogenital ridges (g. ridge) shows expression of a 3-kilobase (kb) *Zfy* transcript specific to adult testis. The upper panel shows hybridization to a 1.9-kb *HindIII* *Zfy-1* genomic fragment containing the zinc-finger encoding exon, which also detects 6- and 8-kb *Zfx* transcripts. The lower panel shows the same filter rehybridized with the first 800 bp of a *Zfy-1* cDNA⁹ which is specific for *Zfy* sequences. **b**, Absence of the 3-kb *Zfy* transcript in XX *Sxr* and XX *Sxr'* adult testes which lack germ cells. The 'zinc-finger' probe described above shows that *Zfx* transcripts are present in both testis types as well as in normal XY testes.

METHODS. Total RNA (10 µg) was electrophoresed on formaldehyde-agarose and transferred to Hybond-N filters (Amersham). Filters were hybridized to ³²P-labelled random-primed DNA probes, then washed in 0.1 × SSC–0.1% SDS at 50 °C for 1 h and exposed to X-ray film for 1–3 days. Parkes outbred mice were used for all experiments unless otherwise stated. The day of vaginal plug was taken to be 0.5 d.p.c. Embryos 9.5–11.5 d.p.c. were sexed by staining for sex chromatin in amnion cells¹⁷, and thereafter by gonadal morphology.

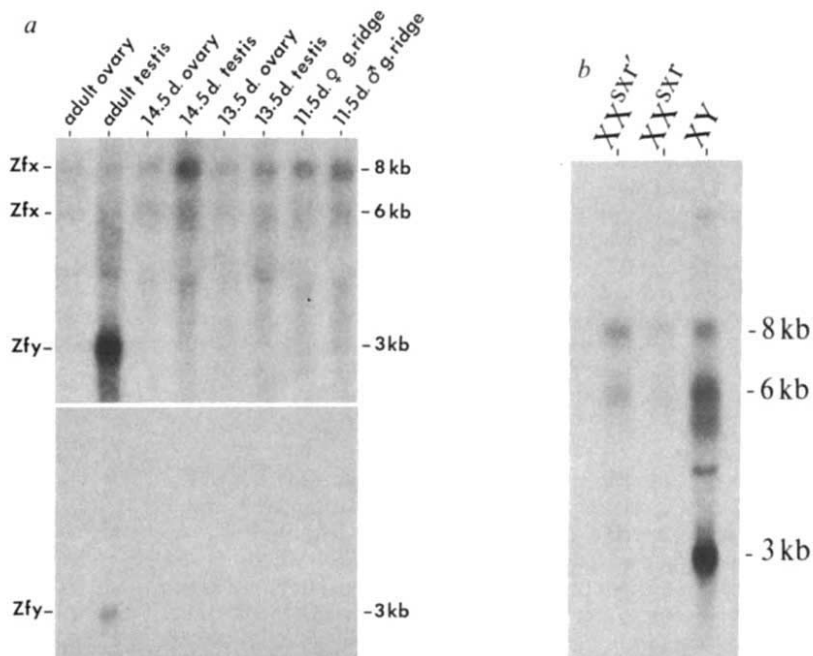
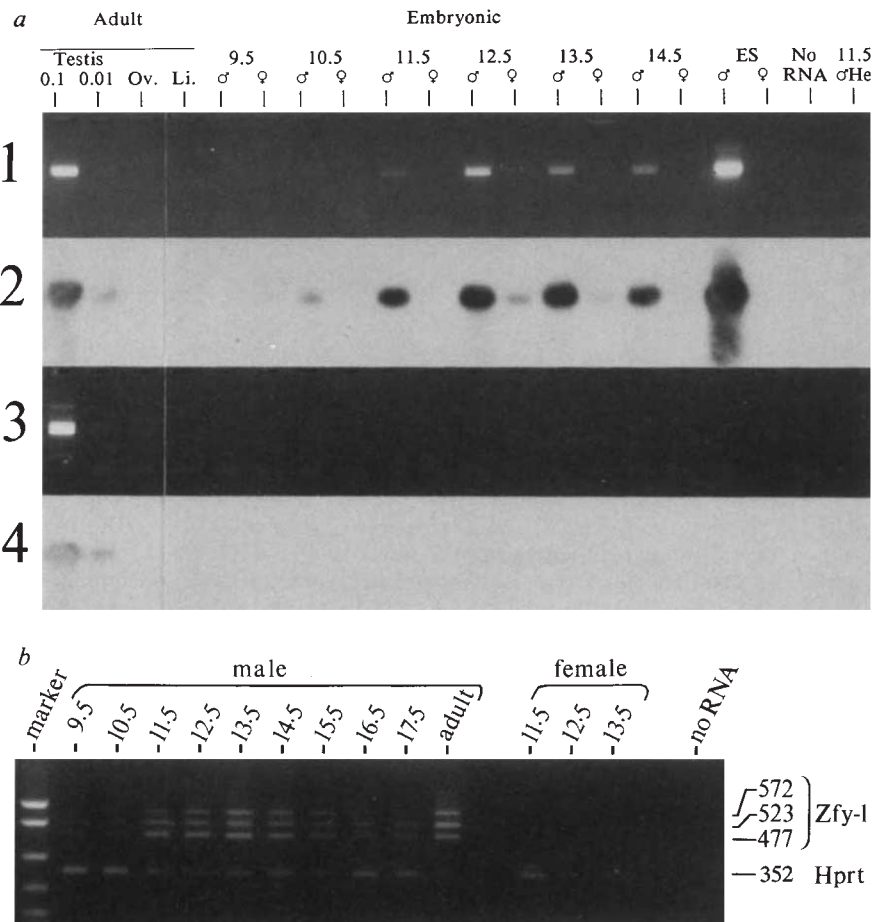


FIG. 2 PCR analysis of *Zfy-1* and *Zfy-2* expression in embryonic mouse gonads. **a**, Bands correspond to *Zfy-1* and *Zfy-2* transcripts in RNA derived from tissue pooled from up to 200 embryos or adults. Rows 1 and 3: ethidium bromide-stained agarose gels showing PCR products obtained using primers specific for *Zfy-1* and *Zfy-2*, respectively. Rows 2 and 4: corresponding Southern blots hybridized with the *Zfy*-specific probe described in Fig. 1 legend. These gels did not allow resolution of the three separate bands associated with *Zfy* expression (see below and Fig. 3). **b**, PCR analysis using RNA prepared from individual embryos or from adult testis. Ethidium bromide-stained agarose gel showing three PCR products obtained using *Zfy-1*-specific primers; specific primers for hypoxanthine phosphoribosyl transferase (*Hprt*) transcripts were included as a positive control for each sample. Band sizes are shown in base pairs. Abbreviations: Ov, adult ovary; Li, adult male liver; 9.5, 9.5-d.p.c. embryos posterior to forelimb bud; 10.5, 10.5-d.p.c. embryos between forelimb and hindlimb buds; 11.5, 11.5-d.p.c. urogenital ridges; 12.5–17.5, 12.5–17.5-d.p.c. fetal gonads; ES, embryonic stem cells, either CCE¹⁸ (♂) or A13¹⁹ (♀); No RNA, water added to reverse transcription reactions in place of RNA solution; 11.5♂He, heads of 11.5-d.p.c. male embryos; Marker, pBR322/*MspI* fragments (622, 527, 404, 309 and 242 bp).

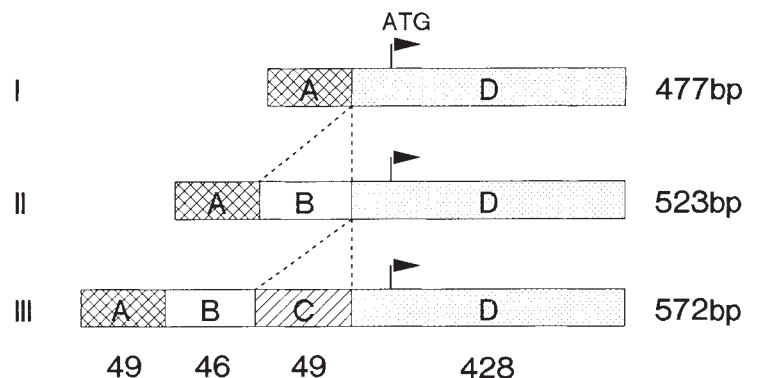
METHODS. **a**, Total RNA (1 µg; 0.1 or 0.01 µg from adult testis) was reverse transcribed in 30 µl as described in ref. 5, at 42 °C for 30 min. Of this, 5 µl was added to a 50-µl PCR reaction containing 0.05% Nonidet P-40, 1.5 mM each dNTP, 500 ng each 'outer' primer, and 2.5 U *Taq* polymerase in the supplied buffer (Anglian Biotec). DNA was amplified by 20 cycles of 94 °C for 5 s, 65 °C for 30 s, and 72 °C for 30 s in a Techne PHC-2. From each reaction, 1 µl was transferred to a new reaction containing 'nested' primers and amplified a further 20 cycles. A 5 µl aliquot was electrophoresed on a 2% agarose-TBE gel. **b**, Small-scale RNA preparations were made using the AGPC method²⁰. The amount of RNA reverse-transcribed corresponded to 100% of 9.5 and 10.5-d.p.c. tissue, 100% of a single 11.5-d.p.c. urogenital ridge or 12.5-d.p.c. gonad, 40%, 20%, 12.5%, 9%, and 7.6% of single testes from 13.5 to 17.5 d.p.c., respectively, 50% of a 13.5-d.p.c. ovary, and 0.5% of an adult testis. These proportions were intended to reflect gonadal volume. In each case the entire 7.5-µl reverse transcription reaction was amplified as above, with the



addition of 500 ng of each *Hprt* primer to the first PCR reaction; 20 µl of the second reaction was electrophoresed. Primer sequences (5'-3') were as follows. *Zfy-1* outer primers were (5') GTTAC TCATT TTCAG GTGTT CTGGG and (3') GTGTC AGCTG TTATA GGATC AGTGA; *Zfy-2* outer primers were (5') CCTGT TGTGT CTGTT GTGGT TCTCG and (3') ATGTC GGCTG TCAAA GGATC AGTGG. These primers were from corresponding regions of the *Zfy-1* and -2 cDNAs^{6,9}. Nested primers are described in Fig. 3 legend. *Hprt* primers were (5') CCTGC TGGAT TACAT TAAAG CACTG and (3') GTCAA GGGCA TATCC AACAA CAAAC from exons 3 and 8 of the mouse gene, respectively²¹.

FIG. 3 Alternative splicing of *Zfy-1* transcripts. The composition of the three different PCR products obtained from 12.5-d.p.c. testis RNA with the *Zfy-1* primers was deduced from DNA sequencing. The nucleotide sequence and size of each block is shown; the translation initiation codon ATG is indicated in block D. Transcript I corresponds to the published *Zfy-1* cDNA sequence⁹, except for the single base difference shown in lower case, presumably a polymorphism between the *Mus musculus musculus* and *M. m. domesticus* subspecies. Transcripts II and III contain the additional exons B and C.

METHODS. PCR as described in Fig. 2 legend; nested primer sequences are shown underlined. DNA from each gel band was cloned into Bluescript (Stratagene) and six clones of each type sequenced using Sequenase (USB).



- A. TGAAGTCTGCAGTACTTGTCTGTCATTGCCTACCCCTCTACACTGGTCTG
- B. ATTATCTGAATAATGTTATAATATGTTCTGACATCATTCTGTTCT
- C. ACATGAATTTATATTTATTATACCTGCTATCAGTTCATTATGGCCCAG
- D. GAGCTGACTTAGTAAGAACTGAAGGCCATGATGAAGATGAAATT
GAATtGACC.....TGCCTGAACATGTCTTGATGAGTGA

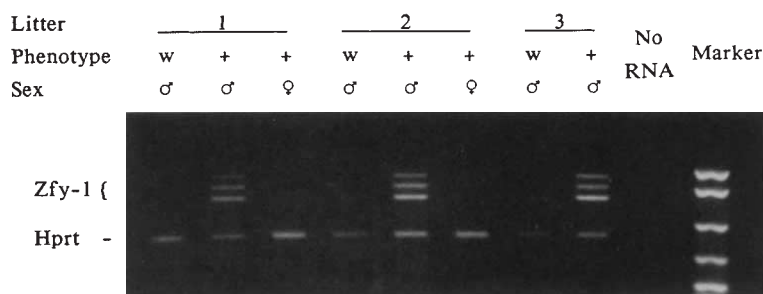


FIG. 4 Lack of *Zfy-1* expression in *W^e/W^e* fetal testes. The three bands corresponding to *Zfy-1* expression were detected using RNA from phenotypically wild-type (+) male fetal testes but not phenotypically mutant (w) testes. The control *Hprt* band was detected in all samples. Littermates from three separate litters were analysed.

METHODS. Random-bred C3H/H-101 *W^e/+* mice were mated and the progeny scored as homozygous mutants by liver morphology at 14.5 d.p.c., (ref. 22) and sexed by gonad morphology. Small-scale RNA preparations were made and analysed as described in Fig. 2 legend. In the first round of the PCR, 50% of the RNA yield from each single gonad was used.

Further, data from XX↔XY chimaeras implies that *Tdy* must act cell-autonomously in the somatic supporting cell lineage of the genital ridge¹⁴. If *Zfy-1* is indeed *Tdy*, expected transcripts of *Zfy-1* should be found in the somatic portion of the developing testis.

To test this, we analysed embryos homozygous for the *W^e* mutation. Their testes lacked *Zfy-1* transcripts (Fig. 4), demonstrating that *Zfy-1* expression in normal testes is due to the presence of germ cells. An alternative pair of PCR primers, from the exons encoding the putative nuclear localization signal and the 'zinc-finger' motifs^{6,9}, confirmed the absence of *Zfy-1* and *Zfy-2* transcripts in *W^e/W^e* fetal testes (data not shown). The formation of testes in the absence of *Zfy* expression, and the association of *Zfy-1* expression with germ cells, argues that *Zfy-1* is not *Tdy*.

ZFY was initially regarded as a candidate for *TDF* by virtue

of its location on the Y chromosome and because its putative product is consistent with cell-autonomous action. Several more recent observations have compromised this view. First, theories of sex determination involving *ZFY*-like genes must take into account the presence of an X-linked homologue in many species and an additional autosomal homologue in mice^{3,5}. Second, *ZFY*-like sequences are not found on the sex chromosomes of marsupials, which seem also to have a Y-dependent sex-determining mechanism¹⁵. Third, *Zfy-1* and *Zfy-2* appear to be expressed normally in a line of mice shown genetically to be mutant in *Tdy* (R.L.-B *et al.*, manuscript in preparation). Fourth, XX male patients have been described who possess Y-linked sequences but not *ZFY*¹⁶. Finally, the sites of expression of *Zfy-1* and *Zfy-2* indicate that neither is *Tdy*, and that both may instead have a role in male germ cell development. □

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Inhibition of endocytic vesicle fusion *in vitro* by the cell-cycle control protein kinase cdc2

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MEMBRANE transport between the endoplasmic reticulum and the plasma membrane, which involves the budding and fusion of carrier vesicles, is inhibited during mitosis in animal cells¹⁻⁵. At the same time, the Golgi complex⁶ and the nuclear envelope⁷, as well as the endoplasmic reticulum in some cell types, become fragmented. Fragmentation of the Golgi is believed to facilitate its equal partitioning between daughter cells⁸. In fact, it has been postulated that both the inhibition of membrane traffic and Golgi fragmentation during mitosis are due to an inhibition of vesicle fusion, while vesicle budding continues⁸. Although less is known

about the endocytic pathway, internalization⁹⁻¹¹ and receptor recycling^{12,13} are also arrested during mitosis. We have now used a cell-free assay to show that the fusion of endocytic vesicles from baby hamster kidney cells is reduced in *Xenopus* mitotic cytosol when compared with interphase cytosol. We reconstituted this inhibition in interphase cytosol by adding a preparation enriched in the starfish homologue of the cdc2 protein kinase. Inhibition was ≥90% when the added cdc2 activity was in the range estimated for that in mitotic *Xenopus* eggs, which indicates that during mitosis the cdc2 kinase mediates an inhibition of endocytic vesicle fusion, and possibly other fusion events in membrane traffic.

To study the regulation of membrane traffic in mitotic cells, we used a cell-free assay that reconstitutes the fusion of early endocytic vesicles. In interphase cells, this fusion apparently reflects lateral interactions between early endosomal elements (for a review, see ref. 14). This fusion may also share an N-ethylmaleimide-sensitive factor¹⁵ and the involvement of GTP-binding proteins¹⁶ with vesicular transport in the secretory pathway¹⁷⁻¹⁹.

As in our previous studies²⁰, the early endocytic vesicles of two baby hamster kidney (BHK) cell populations were labelled separately with avidin and biotinylated horseradish peroxidase (bHRP) by fluid-phase endocytosis for 5 min at 37 °C. Cytosol-free endosomal fractions were then prepared from each cell population, mixed in the fusion assay with cytosol and incubated

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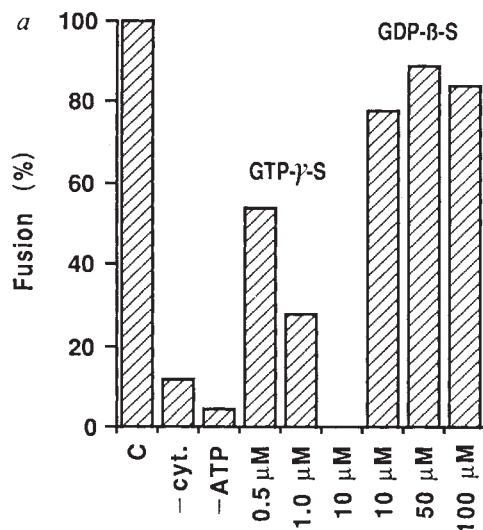
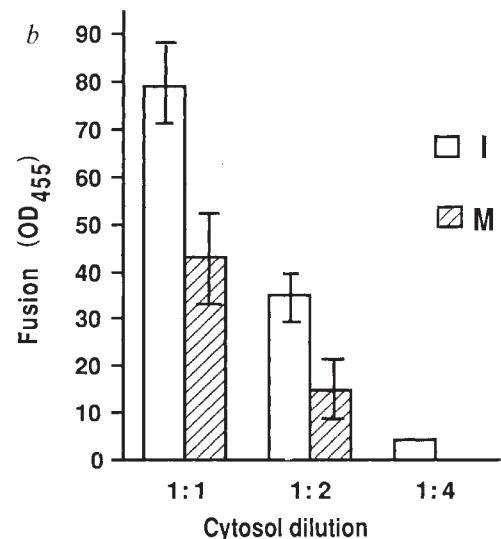


FIG. 1 Early endocytic vesicle fusion reconstituted in *Xenopus* interphase and mitotic cytosols. *a*, To compare different experiments, fusion is expressed as a percentage of the control value. C, control; -cyt., without cytosol; -ATP, without ATP. Concentrations of GTP- γ -S and GDP- β -S as indicated.

METHODS. BHK cells from 15×75 -cm² dishes were released with trypsin, diluted three times with ice-cold PBS containing 5 mg ml⁻¹ BSA (PBS-BSA), centrifuged at 750g for 5 min and washed a further three times in ice-cold PBS-BSA. The cells were then incubated for 5 min at 37 °C with 3.3 mg ml⁻¹ avidin or 1.7 mg ml⁻¹ bHRP in medium²⁰ and washed three times by centrifugation as above. The cells were subsequently resuspended in 10 ml sucrose (0.25 M), 3 mM imidazole pH 7.4 (HB), centrifuged, resuspended in 1 ml HB and homogenized in a tight-fitting Dounce. A post-nuclear supernatant was prepared by centrifugation at 1500g for 15 min, made up to a concentration of 40.6% sucrose, 0.5 mM EDTA in 3 mM imidazole pH 7.4, and loaded in 0.4-ml aliquots at the bottom of a Beckman polycarbonate 11 $\times$ 34-mm tube. The tube was then filled sequentially with a cushion of 0.6 ml sucrose (35%), 3 mM imidazole pH 7.4, 0.5 mM EDTA and 0.3 ml HB, 0.5 mM EDTA. The avidin- (or bHRP-) containing early endocytic vesicles were floated to the 35% sucrose-HB interface by centrifugation for 45 min at 100,000g in

in vitro for 45 min at 37 °C. During the *in vitro* reaction, fusion between avidin- and bHRP-containing endocytic vesicles resulted in the formation of an avidin-bHRP complex. At the end of the reaction, the mixture was extracted in detergents in the presence of excess biotinylated insulin to quench the uncomplexed avidin, and the amount of avidin-bHRP formed was quantitated with enzyme-linked immunosorbent assay (ELISA)^{20,21}. In the studies described here, it was important to use a well-characterized source of mitotic and interphase cytosols. Concentrated extracts (~40 mg per ml protein) were prepared by high-speed centrifugation of *Xenopus* eggs that were either arrested in metaphase II²² or activated by an electric shock²³. The mitotic status of the extracts was estimated by measuring H1 histone kinase activity, and corresponded to 0.5–1.0 pmol of phosphate transferred from ATP to H1 histones min⁻¹ μ l⁻¹ and >8.0 pmol of phosphate transferred from ATP to H1 histones min⁻¹ μ l⁻¹ for interphase and mitotic extracts, respectively. These activities were expected to reflect the cytosolic values *in vivo*²⁴, because the extracts were prepared after a minimal dilution of the egg volume (30% v/v dilution). Fusion of BHK endocytic vesicles could be reconstituted in interphase *Xenopus* cytosol and, as expected, required cytosol as well as ATP (Fig. 1a). The extent of fusion depended on the concentration of added cytosol (Fig. 1b). Specificity of the fusion reaction was shown by its inhibition at low GTP- γ -S concentrations (Fig. 1a). This observation and others¹⁶ indicate that GTP-binding proteins may be involved in controlling endocytic



a table-top TL-100 ultracentrifuge, using a TLS-55 rotor. The interface fraction was collected with a pump, frozen in 25- μ g aliquots in liquid nitrogen and stored until use. Concentrated *Xenopus* interphase and mitotic extracts were prepared as in ref. 25, and contained ~40 mg protein per ml. In the fusion assay, 25 μ g of each fraction (avidin and bHRP) were mixed on ice with 50 μ l cytosol (~2 mg protein). The volume of the reaction was 150 μ l and the mixture contained 50 mM potassium acetate, 12.5 mM HEPES buffer, 1 mM magnesium acetate, 1.5 mM dithiothreitol (DTT), 1 μ g ml⁻¹ cytochalasin D and an ATP-regenerating or -depleting system as described in ref. 20. The fusion reaction was carried out for 45 min at 37 °C in the presence of biotinylated insulin, the samples were then returned to ice-temperature, excess biotinylated insulin added and the samples extracted in detergents²⁰. The fusion-specific complex (avidin-bHRP) was quantitated with ELISA by measuring the enzymatic activity of bHRP at 455 nm (ref. 20). For *b*, The experiment was as for *a*, except that 50 μ l (1:1), 25 μ l (1:2) and 12.5 μ l (1:4) interphase (I) and mitotic (M) extracts were added to the reaction mixture, which was adjusted to 150 μ l, and to the same concentration of reagents as in Fig. 1a. The enzymatic activities of bHRP (optical density (OD) at 455 nm $\times 10^3$) complexed to avidin are indicated for the different extracts.

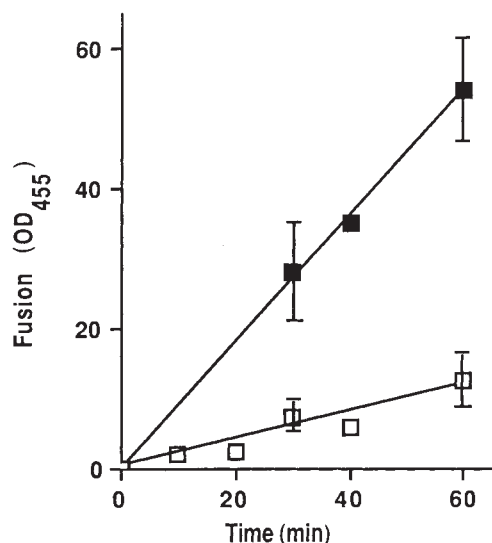


FIG. 2 Time course of the fusion with (□) or without (interphase control, ■) cdc2 kinase. The experiment was as in Fig. 1a, except that 1 μ l cdc2 kinase prepared as in ref. 24 up to the phosphocellulose step, was added to each fraction and to an aliquot of interphase cytosol. For maximal inhibition, each fraction and the cytosol were pre-incubated separately for 15 min at 22 °C in the presence of cdc2 kinase, mixed and then further incubated at 37 °C for the indicated times to allow fusion to proceed. Fusion was measured by bHRP activity at OD₄₅₅ and is indicated as in Fig. 1b.

vesicle fusion. By contrast, high concentrations of GDP- β -S had little effect.

When the assay was carried out in the presence of mitotic cytosol, the fusion activity was reduced (Fig. 1b) and the inhibition observed with different extracts paralleled the H1 histone kinase activities measured during the reaction (see below). This prompted us to test purified preparations of the kinase in the fusion assay. In mitotic *Xenopus* extracts, most of the H1 histone kinase activity is due to the homologue of the fission yeast p34^{cdc2} and the cdc2 kinase from mitotic yeast phosphorylates H1 histones²⁶. The role of the cdc2 kinase in controlling the interphase-mitosis transition in yeast, as in animal cells, is now well established^{27,28}. Using *in vitro* assays, the maturation promoting factor (MPF), which has cdc2 activity, was shown to mediate nuclear envelope breakdown²⁹⁻³¹, chromosome condensation and spindle assembly^{30,31}.

Partially purified preparations of cdc2 kinase were obtained from starfish with high activities corresponding to the transfer from ATP to H1 histone of ~800 pmol phosphate per min per μ l extract. These cdc2 kinase preparations were used to complement the fusion assay in the presence of interphase cytosol. In the absence of cdc2 kinase, fusion increased linearly with the time of the reaction (Fig. 2). When the cdc2 kinase was added at an activity corresponding to 4-5 pmol phosphate transferred per min per μ l reaction mixture, a 75% reduction in the extent of fusion was observed for up to 60 min. Activity of the cdc2 kinase in the assay was also tested using gel electrophoresis and autoradiography by following the phosphorylation of endogenous substrates present in the reaction mixture after 5-min pulses of [³²P]ATP (data not shown).

To establish the relationship between the cdc2 kinase activity and the inhibition of fusion, the assay was performed in the presence of increasing amounts of cdc2 kinase (Fig. 3). This titration correlated well with individual observations made with two different preparations of the cdc2 kinase (Fig. 3). Moreover, the inhibitions observed with mitotic cytosols (see Fig. 1b) were well within the range expected from the H1 histone kinase activities of the corresponding reaction mixtures (Fig. 3). This correlation strongly indicates that inhibition of fusion was mediated by the cdc2 kinase. Fusion activity rapidly decreased as the kinase activity became greater than that corresponding to

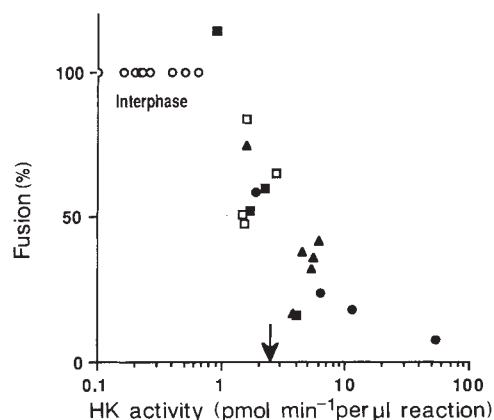


FIG. 3 Titration of fusion at increasing cdc2 kinase activity. Fusion activity is expressed as a percentage of the interphase value so that comparison between different experiments can be made. Titration of the fusion at increasing cdc2 activity (●) is compared with individual experiments carried out with two preparations of cdc2 kinase as in Fig. 2 (■, ▲). □, Assays using mitotic extracts as in Fig. 1b. The H1 histone kinase (HK) activities of the latter reaction mixtures were lower than those of the original mitotic extracts partly because the extracts were diluted three times in the final volume of the reaction (see Fig. 1a). The HK activity was measured as in ref. 25.

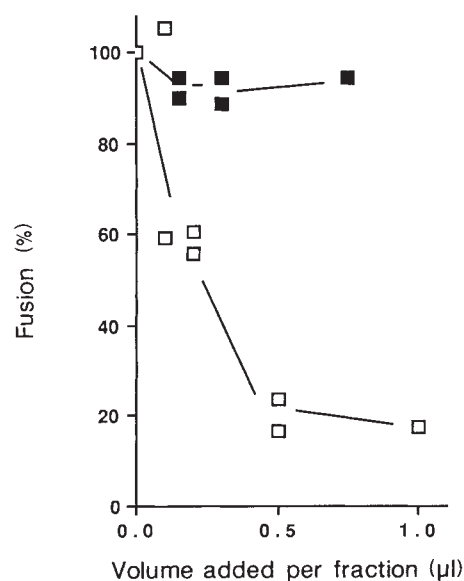


FIG. 4 Depletion of p34^{cdc2} with p13^{suc1} beads. The experiments were carried out as in Fig. 3 after adding the indicated volumes of the cdc2 preparation before (□) or after (■) p34^{cdc2} depletion. Specific and quantitative depletion of the p34^{cdc2} was carried out by affinity chromatography using p13^{suc1} as a ligand³⁴. The p34^{cdc2}-depleted preparations were used in the assay at the same protein concentration as the original p34^{cdc2}-containing preparations.

the transfer of 1.0 pmol phosphate to H1 histones per min per μ l reaction mixture. Half-maximal inhibition was observed at an activity corresponding to the transfer of 2.3 pmol phosphate per min per μ l reaction mixture, and fusion was essentially abolished at activities corresponding to the transfer of ≥ 10 pmol phosphate per min per μ l reaction mixture. Because the kinase activity of the cytosol in mitotic *Xenopus* eggs is estimated to be in this latter range, early endocytic vesicle fusion *in vivo* is probably efficiently arrested during mitosis.

To demonstrate further that fusion inhibition was caused by the cdc2 kinase, the purified starfish preparation was depleted of p34^{cdc2} by affinity-binding to the product of the fission yeast *suc1*⁺, gene p13^{suc1}, coupled to agarose beads. Depletion was efficient and specific, as monitored by H1 histone kinase activity and western blotting with an antibody against the highly conserved PSTAIR amino-acid sequence of p34^{cdc2} (data not shown). The association of p34^{cdc2} with p13^{suc1} (refs 32, 33) has been used to retrieve p34^{cdc2} (refs 34-36). Figure 4 shows that fusion was not inhibited after p34^{cdc2} depletion. Finally, p34^{cdc2} retained on p13^{suc1} beads could be eluted with excess p13^{suc1} and further purified by an additional chromatography step on a mono-S column in a 1:1 stoichiometric complex between p34^{cdc2} and cyclin B (ref. 34). After addition of affinity-purified p34^{cdc2}, a 40% inhibition was observed when the histone kinase activity measured in the assay corresponded to the transfer of 1.5 pmol phosphate per min per μ l. These values agree well with the titration presented in Fig. 3.

These *in vitro* findings strongly indicate that early endocytic vesicle fusion is arrested during mitosis and that this arrest is mediated by the cell-cycle control protein kinase cdc2. Although not directly shown here, this inhibition is expected to be the result of the protein-kinase activity of cdc2. Whether other fusion events are similarly inhibited in mitotic cells, accounting for the observed arrest of intracellular transport, remains to be determined. It is tempting to postulate that cdc2 kinase is part of the central regulatory mechanism controlling membrane traffic in mitotic cells. □

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Dissection of functional domains of the pituitary-specific transcription factor GHF-1

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THE specific expression of growth hormone (GH) in the somatotrophic cells of the anterior pituitary is largely attributable to a short promoter in the 5' flanking region of the GH gene^{1–6}. This promoter contains two binding sites for the transcription factor GHF-1 (refs 3,7), the expression of which is also specific to cells of the somatotrophic lineage^{8,9} and correlates with activation of the GH gene in the developing mouse pituitary⁹. Various studies indicate that GHF-1 is the main determinant of cell type-specific expression of the GH gene^{3–9}. GHF-1 is a member of the POU-domain class of proteins that each contain two highly conserved sequence motifs, the homeodomain and the POU-specific domain^{8,10,11}. Here we report that the GHF-1 homeodomain is sufficient for sequence-specific DNA binding, although its activity is stimulated by the POU-specific domain, which does not interact directly with the DNA. Transcriptional activation is mediated by a separate domain rich in hydroxylated amino-acid residues. Similar sequences are present in other cell type-specific transcription factors.

To examine the ability of GHF-1 to specifically activate the GH gene promoter, HeLa cell lines were generated containing a stably integrated human metallothionein-II_A-GHF-1 (*MT-GHF1*) fusion gene in which expression and nuclear accumulation of GHF-1 were dependent on Cd²⁺, an activator of the human metallothionein-II_A gene (Fig. 1a). As judged by indirect immunofluorescence, these cells express nearly as much GHF-1 as pituitary-derived cells⁸. In HeLa *MT-GHF1* cells, expression

of a transfected reporter construct containing a fusion of the GH gene and the chloramphenicol acetyltransferase (CAT) gene (*GH-CAT*) was strictly dependent on induction of *MT-GHF1* by Cd²⁺ (Fig. 1b). A construct containing a mutant GH gene promoter lacking GHF-1 binding sites, *GHΔ*(–82/–128-CAT), was refractory to Cd²⁺, as was a construct containing a prolactin gene (*Prl*) linked to a CAT gene (*Prl-CAT*). None of these constructs was inducible by Cd²⁺ in wild-type HeLa cells (data not shown). RNA analysis confirmed that the observed activation was transcriptional (data not shown).

These results indicate that GHF-1 is a specific activator of GH gene expression. No significant activation of the *Prl* promoter was observed, confirming that GHF-1 has a much higher affinity for the GH gene promoter than does a second factor, LSF-1, which binds more efficiently to the *Prl* promoter¹³. Our experiments enabled better control over the level of GHF-1 expression than that obtained in the experiments of Ingraham *et al.*, which showed that transiently transfected GHF-1 activates both promoters¹⁰. In addition, these authors used a construct which also contained the distal enhancer of the *Prl* gene, whereas we directly compared the *Prl* and GH gene promoters in the absence of upstream enhancers. Although the promoter region is sufficient for activating the GH gene in response to GHF-1,

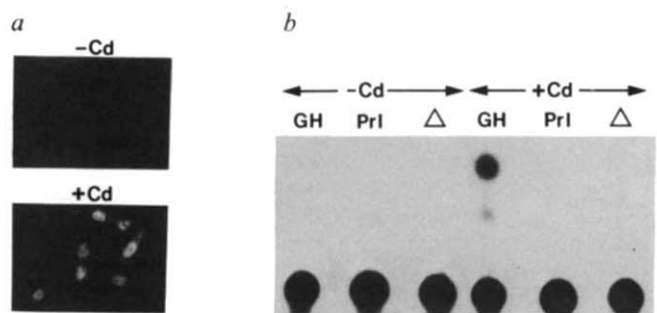


FIG. 1 GHF-1-dependent activation of *GH-CAT* in HeLa cells. *a*, Inducible expression of GHF-1 in HeLa cells stably transfected with *MT-GHF1*. HeLa *GHF-1*⁺ cells were incubated in the presence or absence of 2×10^{-6} M CdCl₂ for 16 h, fixed and analysed by indirect immunofluorescence with anti-GHF-1 antibodies. *b*, Specific activation of the GH gene promoter after induction with Cd²⁺. HeLa *GHF-1*⁺ cells were transfected with *GH-CAT* (GH), *Prl-CAT* (Prl), or *GHΔ*(–82/–128)-CAT (Δ , a mutant GH gene promoter lacking GHF-1-binding sites). After transfection, cells were incubated for 16 h in the absence (–) or presence (+) of 2×10^{-6} M of CdCl₂ and CAT activity was determined. Results of one representative assay are shown. METHODS. A fragment containing the entire coding region of rat GHF-1 gene, flanked by appropriate restriction sites, was obtained by the polymerase chain reaction (PCR)³³ using λ gt10 clone 2A, which contains a full-length GHF-1 complementary DNA⁸, as a template. Oligonucleotides complementary to the regions of translation initiation and termination and also containing *Hind*III and *Not*I restriction sites were used as primers. The 920-base pair (bp) GHF-1 fragment was digested by *Hind*III and *Not*I and inserted into the expression vector RSVc-Jun¹⁷, in place of the *c-Jun* coding sequence. The authenticity of this construct (*RSV-GHF-1*) was confirmed by sequencing both strands³⁴. Plasmid pMT-GHF-1 was constructed by cloning a 1.76-kilobase (kb) *Bam*HI fragment derived from *RSV-GHF-1* into plasmid pHSI, which contains human metallothionein IIA sequences from position +76 to –764 (ref. 12). HeLa cells were co-transfected with 15 μ g plasmid pMT-GHF-1 and 3 μ g plasmid pRSV-Neo by the Chen–Okayama procedure³⁵ and selected for G418 resistance. Individual colonies were isolated and analysed for Cd²⁺-inducible GHF-1 expression by indirect immunofluorescence⁸. Plasmid pRL-CAT contains the 415-bp *Hind*III–*Pst*I *Prl* gene promoter fragment (–9 to –423; ref. 13); plasmid pGH-CAT and plasmid pGH Δ (–82/–128)-CAT were previously described³ and contain human GH gene promoter sequences from position +1 to –289. The complete sequences of all promoter regions used in these constructs were confirmed by sequencing both strands. Transient transfections were done with 2.5 μ g of the various CAT-containing reporter constructs, 5 μ g *RSV-LucA3* (ref. 36) and 12.5 μ g carrier DNA. CAT and Luciferase activities were determined as previously described^{3,36}. Luciferase activity was used as an internal control to judge the uniformity of the transfections.

activation of *Prl* may require synergistic interactions with the enhancer region. Similar results were observed in transgenic mice⁶.

To determine the structural features of GHF-1 responsible for its activity as a transcription factor, a series of internal deletion mutants was generated (Fig. 2). The ability of these mutants to activate the GH gene promoter was examined by a co-transfection assay in both F9 and Rat-6 cells. Although the extent of GH gene promoter activation in this assay was not as high as that observed in HeLa *MT-GHF1*⁺ cells, because of a higher basal level, it was more convenient for screening a large number of mutant proteins. In this assay, activation of the GH gene promoter was also dependent on the presence of GHF-1-binding sites (data not shown).

Three different regions are required for efficient activation of *GH-CAT* by GHF-1. Deletion of amino-acid residues 2–45 and 48–73, within a serine-threonine-rich region, led to a large decrease in activity. Similarly, deletion of residues 124–201 encompassing the POU-specific domain, and residues 209–252 and 255–291 within the homoeodomain, had a strong deleterious effect. By contrast, deletion of residues 72–125 containing a cluster of negatively charged residues, and residues 200–211 constituting a putative hinge region between the POU-specific domain and the homoeodomain, did not impair activity. In fact, GHF-1 with a deletion of residues 72–125 (mutant $\Delta 72-125$) was more active than wild-type GHF-1. All of the mutants, with the exception of $\Delta 125-201$, which could not be tested because of the loss of the antigenic epitope, and $\Delta 211-254$, were efficiently expressed and accumulated in the nucleus (Fig. 2). Mutant $\Delta 211-254$ lacking the N-terminal half of the homoeodomain exhibited diffuse cytoplasmic fluorescence (data not shown) indicating that the nuclear translocation signal of GHF-1 is located within the deleted region.

The homoeodomain is the minimal DNA-binding domain of several proteins^{14–16}. To analyse the DNA-binding domain of

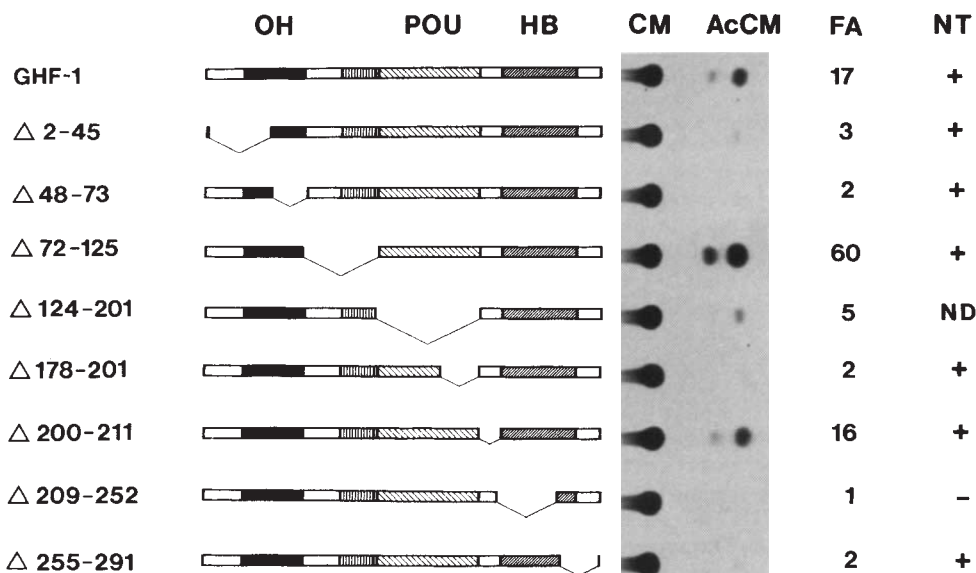
GHF-1, two chimaeric proteins were constructed, Jun-G123 and Jun-G202, by fusing the activation domain of c-Jun¹⁷ to C-terminal GHF-1 sequences either containing the POU-specific domain (residues 123–291) or lacking the POU-specific domain (residues 202–291). Both chimaeras specifically activated *GH-CAT* expression; the one containing the POU-specific domain was fivefold more active than the one that lacked this region (Fig. 3a). The role of the POU-specific domain in DNA binding was also investigated by preparing two TrpE-GHF-1 fusion proteins. One, TrpE-GHF-1, contained the entire GHF-1 sequence (residues 1–291), whereas the other, TrpE-GHF(HB), contained the entire homoeodomain and only the C-terminal half of the POU-specific domain (residues 164–291; Fig. 3b). Footprinting (Fig. 3c) and mobility-shift (data not shown) experiments indicated that the protein containing the entire POU-specific domain exhibits 2.5- to 3-fold higher relative affinity than the protein lacking half of this motif. Most importantly, binding of the two proteins resulted in very similar DNase I protection patterns. As shown previously⁸, these footprints were essentially identical to those generated by native GHF-1 purified from pituitary cells.

Although TrpE-GHF(HB) also lacks the N-terminal half of the protein, taken collectively, these results strongly indicate that the POU-specific domain of GHF-1 is not directly involved in making contact with the DNA, but somehow potentiates binding by the homoeodomain, which, as in other members of this family^{14–16}, is the minimal DNA-binding domain. It is possible that the POU-specific domain is responsible for increasing the binding specificity of GHF-1, because high concentrations of TrpE-GHF(HB) protect additional sequences not recognized by native GHF-1 (ref. 8). One possible explanation for the difference between our results and those of Sturm and Herr¹⁸ is that, unlike GHF-1, Oct-1 could be more dependent on the POU-specific domain because of the lower intrinsic DNA-binding affinity of its homoeodomain.

FIG. 2 Mutational analysis of GHF-1.

The structure of GHF-1 and the internal deletion mutants are shown in a schematic form. Hatched boxes, homoeodomains (HB) and POU-specific domains (POU); striped boxes, regions rich in negatively charged residues; solid black boxes, regions rich in hydroxylated amino acids (OH); and open boxes, remainder of protein. Thin lines denote deleted sequences. Mutants are named according to the sequences affected by the deletions. The activities of the various constructs were tested by co-transfection with the *GH-CAT* reporter gene and determination of CAT activity. Results of one typical assay using Rat-6 cells are shown next to the diagram outlining the various constructs. Abbreviations: CM, chloramphenicol; acCM, acetylated chloroamphenicol. Fold activation (FA) is the activity of the various constructs relative to that of $\Delta 209-252$ which does not activate the GH gene promoter. Similar results were seen in two other assays in both Rat-6 and F9 cells. No activation of the construct containing the mutant GH gene promoter, *GH* $\Delta(-82/-128)$ -*CAT* (Fig. 1) was observed (data not shown). Nuclear transfer (NT) of the various proteins was determined by indirect immunofluorescence using an antibody specific for GHF-1. ND, not detected. These assays also served as controls for the quality of the transfections. Levels of GHF-1 expression were similar for all of the constructs indicated as NT positive (+).

METHODS. The *RSV-GHF-1* expression vector described in Fig. 1 was used as a template for generating by PCR³³ the various deletion mutants. Mutagenic primers were complementary to the sequences flanking the



deleted regions such that one series of reactions generated 5' fragments and the other 3' fragments. These fragments were joined together to yield the final constructs, whose identity was confirmed by sequencing³⁴. *GH-CAT* (5 μ g) were transfected into Rat-6 or F9 cells on 10-cm plates together with 10 μ g of the various expression vectors and 5 μ g of carrier DNA, and CAT activity was determined 40 h later. Similar results were obtained in both F9 and Rat-6 cells. For immunofluorescence analysis, F9 cells were grown on cover slides and transfected as described above. Cells were fixed 10 h after removal of DNA precipitate and examined by indirect immunofluorescence with anti-GHF-1 antibodies⁸.

Because the N-terminal half of GHF-1 is not involved in DNA binding, deletions of residues 2-45 and 48-73 probably affect the transcriptional activation domain. It is of interest that residues 1-73 contain only three sparsely spaced negatively charged residues, whereas residues 72-125, the deletion of which did not impair function, contain most (10 out of 13) of the negatively charged residues within the N-terminal domain. Therefore, residues 1-73 could constitute a novel activation domain that is different from the well-characterized negatively charged activation domains found in a variety of yeast and mammalian proteins^{17,19-21}. Fusion of different portions of GHF-1 to the c-Jun DNA-binding domain, which recognizes the AP-1 binding site^{17,22}, confirmed these assertions. Despite possible interactions with endogenous Jun and Fos proteins, the c-Jun DNA-binding domain of the mutant protein JunΔ6-223 is practically inactive¹⁷. Fusion of GHF-1 residues 1-71 was sufficient to confer transcriptional stimulatory activity on the c-Jun DNA-binding domain (Fig. 4a). These experiments also indicate that residues 71-208 contribute very little to this activity. The primary sequence of the GHF-1 activation domain is shown in Fig. 4b.

The results described above demonstrate that the pituitary-specific transcription factor, GHF-1, is composed of two functional domains. The C-terminal half of this protein of relative molecular mass 33,000 (M_r 33K) contains its DNA-binding domain, whereas the N-terminal half is involved in *trans*-activation. Like other homeodomain proteins¹⁴⁻¹⁶, the minimal DNA-binding domain of GHF-1 is composed of its homeodomain. The N-terminal portion of the homeodomain is also likely to contain the nuclear transfer signal, a good candidate for which is a stretch of six contiguous basic residues between positions 213 and 219, similar to other nuclear transfer signals²³. In addition to the homeodomain, optimal DNA-binding requires the POU-specific domain. But, the exact part played by this motif is not clear because it does not seem to be involved in making contact with the specific binding sites for GHF-1. It is possible that the POU-specific domain stabilizes the protein-

DNA complex and increases binding specificity by a so far unknown mechanism.

The N-terminal half of GHF-1 is responsible for *trans* activation. Construction of GHF-1-Jun chimaeric proteins enabled the demonstration that the first 71 amino-acid residues of GHF-1 function as an independent activation domain. This part of the protein contains only three sparsely spaced negatively charged residues and two glutamines (Fig. 4b). Therefore it bears no primary sequence homology to the two prototypes of activation domains described previously^{19-21,24}. By contrast, more than 30% of the amino and residues in this region of GHF-1 are hydroxylated residues: serine, threonine and tyrosine. Because of phosphorylation, this region could constitute an acidic activator²¹. But immunoprecipitation of ³⁵S- and ³²P-labelled GHF-1 indicates that the extent of GHF-1 phosphorylation is very low (L. H. K. Defize, unpublished results). In addition, competition experiments indicate that the GHF-1 fails to compete against the acidic activation domain of c-Jun, whereas homologous competition is very efficient (unpublished results). We conclude therefore that the activation domain of GHF-1 represents a new class of activation domains.

So far the essential structural features of this domain are not known. The most remarkable feature of this part of GHF-1, however, is the abundance of hydroxylated amino acids. (Fig. 4b) and therefore, we have named it the STA domain (Ser-Thr-rich activation domain). It is noteworthy that two other members of the POU-domain family, Oct-2 and Unc-86, both of which are cell type-specific activators, contain regions that are abundant (>30%) in hydroxylated amino acids. In Oct-2 this region is located near its C terminus²⁵⁻²⁷, and in Unc-86 it is located immediately upstream of the POU-domain²⁸. Two other cell type-specific *trans*-acting factors that do not belong to the homeodomain family, MyoD1 and Myf-5, also contain Ser-Thr-rich regions²⁹. At least for Oct-2, this region could be important for *trans*-activation (W. Herr, personal communication). Although Ser-Thr-rich regions are also present in Sp1 they are dispensable for transcriptional activation in *Drosophila* cells²⁴;

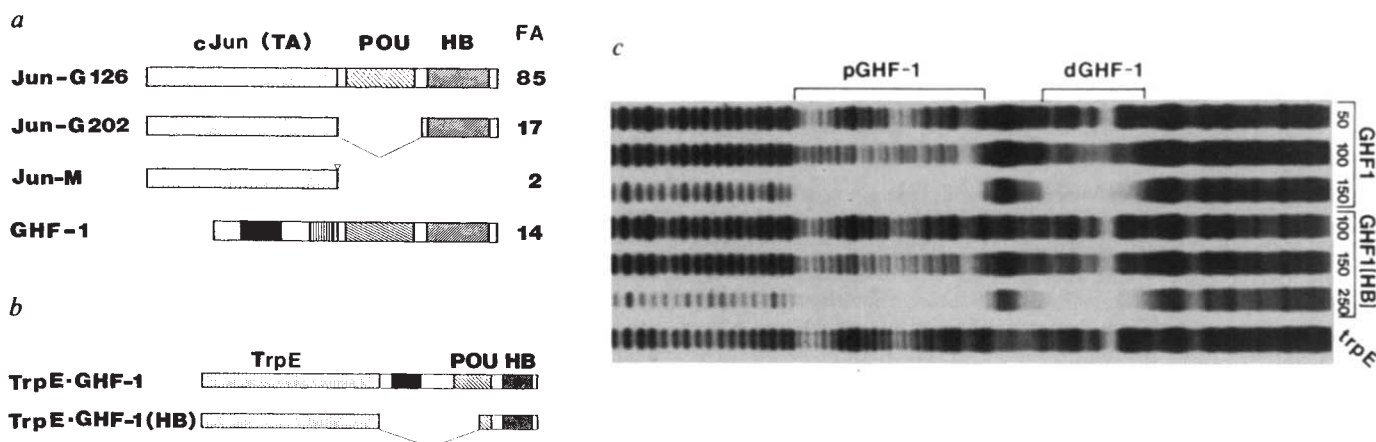


FIG. 3 Role of the POU-specific domain. **a**, Structure and activities of chimaeric Jun-GHF-1 constructs. Dotted box, c-Jun activation region (amino-acid residues 1-193); other symbols denote GHF-1 sequences as described in (Fig. 2). The activities of these constructs were determined by transfection of F9 cells with 10 μ g of expression vector and 5 μ g *GH-CAT* or *GHA*(-82/-128)-*CAT* lacking GHF-1-binding sites. Basal *GH-CAT* expression was determined by co-transfection with RSVcJunΔ6-223, which lacks the activation region¹⁷ and was given an arbitrary value of 1. Fold activation (FA) was determined relative to this value and represents the average of two different experiments. Expression of *GHA*(-82/-128)-*CAT* was not affected by any of the expression vectors (data not shown). **b**, Structure of the TrpE-GHF-1 and TrpE-GHF(HB) fusion proteins. Dotted box, TrpE N-terminal region (M_r 37K); other boxes denote GHF-1 sequences as described above. **c**, Footprint analysis of the DNA-binding specificities of TrpE-GHF-1 and TrpE-GHF(HB). End-labelled human GH gene promoter fragment (+3 to -500; labelled at +3) was incubated with increasing amounts

(ng) of the two fusion proteins as indicated, or 400 ng plasmid p 37^{trpE} and subjected to DNase I footprinting.

METHODS. To construct Jun-G126 and Jun-G202, *Bgl*II-*Asp*718 DNA fragments from Δ72-125 and Δ124-201 (containing the sequences encoding the GHF-1 POU-HB and HB, respectively) were inserted into *c-Jun*Δ194-223 (ref. 17) replacing the c-Jun DNA-binding domain. c-Jun-M contains the c-Jun activation domain (amino-acid residues 1-193) and lacks the DNA-binding domain¹⁷. Transfections were as described above. TrpE-GHF-1 was constructed by inserting a 0.9-kb *Bam*HI-*Hind*III fragment containing the complete GHF-1 gene coding region into the polylinker region of plasmid pATH3 which contains the TrpE N-terminal-encoding region, coding for p37^{trpE} (ref. 22). *trpE*-GHF(HB) contains an *Eco*RI fragment corresponding to GHF-1 amino-acid residues 164-291 as previously described⁸. Preparation of TrpE fusion proteins, gel-retardation assays (not shown), and DNase I footprinting reactions as previously described⁸. Amounts of proteins were determined by silver staining.

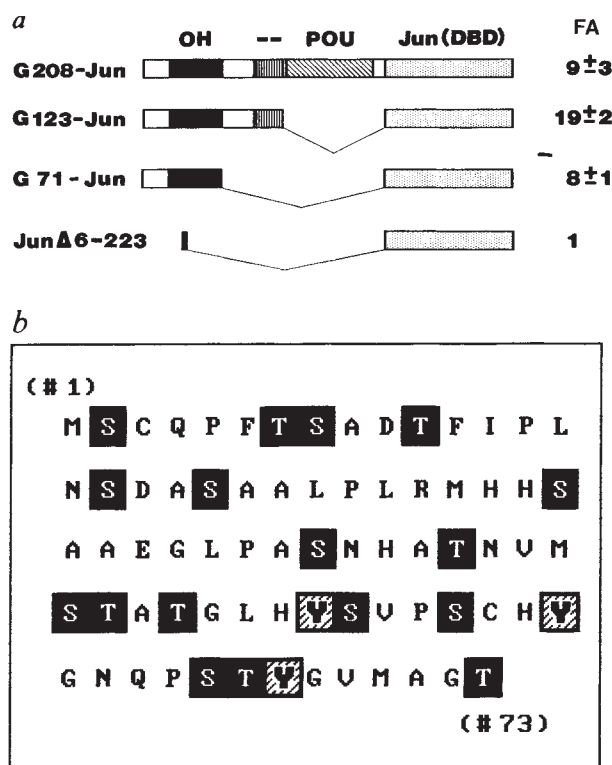


FIG. 4 GHF-1 contains a novel activation domain. *a*, Structures and activities of chimaeric constructs, containing N-terminal fragments of GHF-1 fused to the DNA-binding domain of c-Jun (dotted box). The activities of these constructs were determined by transfections of F9 cells with 10 µg expression vector and 3 µg of -73-Col-CAT, which contains an AP-1-binding site^{17,22} and assaying CAT activity 20 h later. Fold activation (FA) is relative to the basal expression of -73-Col-CAT, which was determined by co-transfection with JunΔ6-223 (ref. 17) and represents the average of three separate experiments. *b*, Primary structure of the GHF-1 activation domain. The serine, threonine and tyrosine residues are highlighted (single-letter amino-acid code).

METHODS. To construct G208-Jun, G123-Jun and G71-Jun, a 1.6-kb BglII-Asp718 DNA fragment corresponding to the c-Jun DNA-binding domain was isolated from RSVcJunΔ194-223 (ref. 17) and inserted in-frame into Δ209-252, Δ124-201 and Δ72-125, respectively.

but it is possible that in other cell types these regions are active. It is of interest that the STA domain has a similar composition to the C-terminal repeat of the largest subunit of RNA polymerase II (ref. 30). In addition to hydroxylated residues, these regions contain several prolines, indicating that they do not form α-helices. It is possible that the STA domain may contact directly, by hydrogen bonding, the C-terminal domain of the largest subunit of RNA polymerase II. Because the C-terminal domain of the largest subunit is subject to phosphorylation³¹, it is more likely to make contact with the hydroxylated or basic amino-acid residues than acidic residues as previously suggested³². □

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A highly divergent HIV-2-related isolate

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It has been suggested that the human immunodeficiency virus type 2 (HIV-2) and the simian immunodeficiency virus from rhesus macaques (SIV_{mac}) evolved from the sooty mangabey virus SIV_{sm} (ref. 1). We now describe an HIV-2-related isolate, HIV-2_{D205}, from a healthy Ghanaian woman that is genetically equidistant to the prototypic HIV-2 strains and to SIV_{sm} and SIV_{mac}. Supported by the observation that HIV-2_{D205} differs in a step of envelope glycoprotein processing, our data indicate that it could represent an alternative HIV-2 subtype and that viruses of the HIV-2/SIV_{sm}/SIV_{mac} group could have already infected humans before HIV-2 and SIV_{sm}/SIV_{mac} diverged.

We have previously reported the molecular cloning of HIV-2_{D205} (ref. 2). This isolate from a healthy woman from Ghana was biologically characterized by its good growth on macrophages and by the absence of cytopathic effects on lymphocytes. It was originally classified as HIV-2 by western blot analysis.

We have now determined the complete nucleotide sequence of the clone HIV-2_{D205,7}, which encompasses 7,817 base pairs (bp) of the proviral genome starting from the left long terminal repeat (LTR). We compared the sequence with the known HIV and SIV sequences (Table 1). Surprisingly, HIV-2_{D205,7} is only 76.2-76.8% identical to the other HIV-2 strains sequenced so far (strains ROD³, D194², NIH2⁴ and ISY⁵). But the relationship of HIV-2_{D205,7} to SIV_{sm} (ref. 1; 76.4% nucleotide-sequence identity), SIV_{mac} (ref. 6; 75.0%), SIV_{agm} (ref. 7; 58.2%) and HIV-1_{BRU} (ref. 8; 57.8%) is in the same range as for the other HIV-2 isolates. HIV-2_{D205} is, therefore, genetically equidistant to the other HIV-2 strains and to SIV_{sm} and SIV_{mac}. For comparison, the identity between the other HIV-2 strains is 87-90%, their identity to SIV_{sm} is 76.5-77.7%, and that to SIV_{mac} is 75.3-76.3%.

The genetic organization of HIV-2_{D205} is the same as for HIV-2, SIV_{sm} and SIV_{mac}. Many of the open reading frames (*orf*) of HIV-2_{D205} differ in length only by one or a few codons from the *orf* of HIV-2_{ROD}. The most notable difference is a

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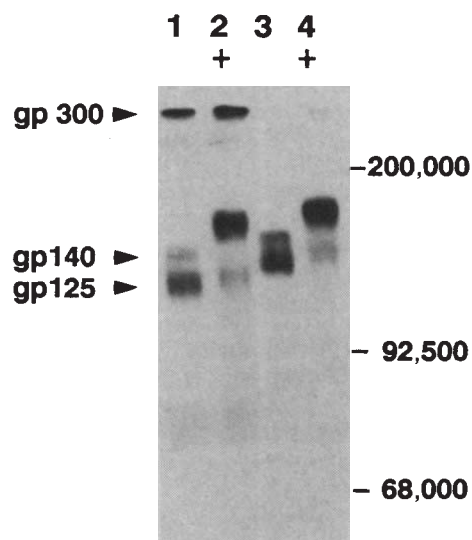


FIG. 1 Size comparison of the external glycoproteins (gp) of HIV-2_{ROD} and HIV-2_{D205}. [³⁵S]cysteine-labelled proteins from cells infected with HIV-2_{ROD} (lanes 1, 2) and HIV-2_{D205} (lanes 3, 4), immunoprecipitated by HIV-2-positive serum, and separated on an 8.5% SDS polyacrylamide gel. The external gp of HIV-2_{ROD} (gp125), its precursor (gp140), the dimeric form of gp140 (gp300), and the positions of *M_r* markers are indicated. In HIV-2_{D205}-infected cells, the band corresponding to the dimer is missing, even after treatment with castanospermine (lane 4), an inhibitor of glucosidase I, which causes the dimeric form to accumulate in HIV-2_{ROD} (lane 2). Notice that the external gp and its precursor are larger in HIV-2_{D205} than in HIV-2_{ROD}.

54-bp insertion in the *gag-pol* overlap, followed by a 54-bp deletion, which puts the codons in phase again. This deletion, but not the insertion, is also present in the *gag-pol* *orfs* of SIV_{sm} and SIV_{mac}. In addition, the *gag-pol* overlap of HIV-2_{D205} is 60 nucleotides longer than it is in HIV-2_{ROD}, resulting in a *pol* *orf* that is extended by 20 codons at the 5' end. We found some minor *orfs* by examining the DNA (+) strand. One of them, at position 1,851–1,651 on the lower strand of the HIV-2_{D205,7} sequence, is conserved between all HIV-2 isolates, as well as in SIV_{mac} and SIV_{sm} (data not shown), and was preliminarily named *orf-y*. We predicted the deduced amino-acid sequence to constitute a small very hydrophobic protein of relative molecular mass 7,300 (*M_r* 7.3). Although there is, so far, no evidence for the transcription of the DNA (+) strand, of retroviruses, its remarkable conservation implies that it could have a function.

We compared the sequences of single genetic units from HIV-2_{D205}, HIV-2_{ROD}, SIV_{sm} and SIV_{mac}, and found that variability ranges from 4.6% (for genetic unit *R*) up to 35.9% (for *rev* exon 1) on the nucleotide level, and from 12.8% (for *gag*) up to 47.6% (for *rev* exon 1) at the amino-acid level (Table 2). But functionally relevant sequences or protein characteristics (such as hydrophilicity or charge) are highly conserved. For example, the identity of the complete LTR of HIV-2_{D205} to that of HIV-2_{ROD} is 84.5%, whereas the identity for TAR, the region necessary for the *trans*-activation by Tat^{9,10}, or the binding site

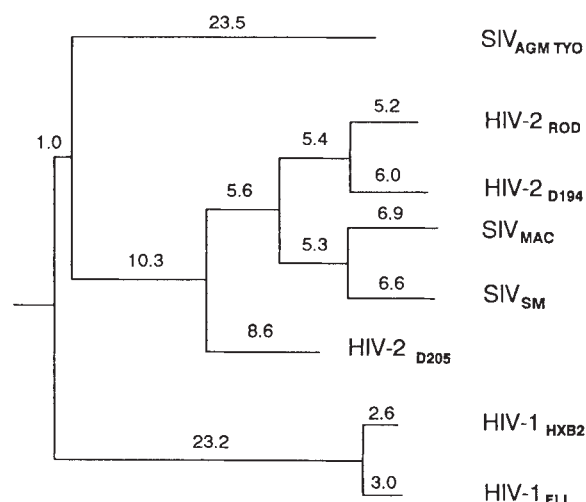


FIG. 2 Minimum-length evolutionary tree showing the relationship of HIV-2_{D205} to the HIV and SIV lentiviruses. The tree was generated from single base substitutions in the 3' part of the HIV-2_{D205,7} sequence using the PAUP program¹³ and version 3.21 of the PHYLIP¹⁴ bootstrapping algorithm. The tree was rooted on a random sequence. The total number of sites examined was 2,664; total number of variable sites, 1,548; consistency index, 0.746. Horizontal distances are proportional to the minimum number of single nucleotide substitutions required to generate the sequence examined or to the actual branch lengths indicated as fractional steps, that is, percentage of total sites.

for the transcription factor NF- κ B (ref. 11), is 100%. The primer-binding site is 100% complementary to the 3' end of the transfer RNA for lysine and the 'shifty sequence' TTTTTT, which catalyses the ribosomal frameshift between the *gag* and *pol* *orfs*, is unaltered. The sequenced part of the external glycoprotein of HIV-2_{D205} is 33% divergent from that of HIV-2_{ROD}, but has the same pattern of conserved and variable domains as the other HIV-2 and SIV_{sm}/SIV_{mac} strains¹⁰. All cysteine residues in the external glycoprotein of HIV-2_{ROD} are conserved. Divergence in the external glycoproteins is due to single amino-acid exchanges and small deletions or insertions, mainly in the variable regions. In addition, 8 out of 21 potential N-glycosylation signals differ between HIV-2_{D205} and HIV-2_{ROD}, indicating the importance of glycosylation in the generation of envelope variability.

To further characterize HIV-2_{D205}, we immunoprecipitated viral proteins and analysed them by SDS-PAGE. It is noteworthy that the envelope precursor glycoprotein, which forms a dimer in HIV-2 and SIV_{mac} to be processed correctly¹², was not dimeric in HIV-2_{D205} (Fig. 1). Furthermore, the external glycoproteins of HIV-2_{D205} and its precursor have higher apparent *M_s* than those of HIV-2_{ROD}. This difference was also observed when glycosylation was inhibited (M.A., manuscript in preparation).

To determine the position of HIV-2_{D205} in the phylogenetic tree of lentiviruses, we constructed minimum-length evolutionary trees¹³. The tree based on the nucleotide variation in the 3' part of the HIV-2_{D205,7} sequence is shown in Fig. 2. HIV-2_{D205} is located remarkably close (8.6% difference) to a hypothetical

TABLE 1 Nucleotide sequence identity between HIV and SIV viruses (in %)

Strain	HIV-2				sm	SIV mac	AGM	HIV-1 BRU
	ROD	D194	NIHZ	ISY				
HIV-2 _{D205,7}	76.8	76.2	76.4	76.7	76.4	75.0	58.2	57.8
HIV-2 _{ROD}	—	87.0	90.1	89.3	77.7	76.3	58.0	57.0
SIV _{mac251}	76.3	75.3	76.4	75.8	84.6	—	58.7	55.0

Sequences were obtained from the Los Alamos HIV Sequence Database¹⁰ and compared using Microgenie sequence software from Beckman.

common ancestor of the HIV-2/SIV_{sm}/SIV_{mac} group of lentiviruses and branches before SIV_{sm}/SIV_{mac} diverged from the HIV-2 prototypes. In the tree, HIV-2_{D205} is 25% different from the prototypic HIV-2 viruses and 26% different from SIV_{sm} and SIV_{mac}, which agrees with our nucleotide-sequence comparison data in Table 1. This tree was congruent with the majority tree for the 5' half of the genome. Bootstrapping, using version 3.21 of the PHYLIP algorithm¹⁴, led to the same branching order in 40 out of 50 replicates. Branching length and branching order in this tree were in good agreement with those of other work (for example, see Fig. 4 of ref. 1). In the evolutionary tree analyses, the rarest occurrence was the clustering of HIV-2_{D205} with the prototypic HIV-2 viruses; sometimes HIV-2_{D205} clustered with SIV_{sm}/SIV_{mac}, but it usually appeared by itself as a close relative of the progenitor of both.

Our data indicate that the HIV-2/SIV_{sm}/SIV_{mac} group of viruses is older and genetically more divergent than previously believed. This antiquity, along with a high degree of variability, presumably accounts for the development of different subtypes: the HIV-2 prototypes, an alternative subtype defined by the strain HIV-2_{D205}, described here, and the simian strains SIV_{sm} and SIV_{mac}. The alternative subtype defined by HIV-2_{D205} is characterized by the high sequence divergence (24%) from that of the HIV-2 prototypes, and by the absence of dimer formation of the envelope protein precursor (Fig. 1), which is believed to be a necessary step in the processing of the envelope glycoproteins in the prototypic HIV-2 viruses and in SIV_{mac}. In this respect, HIV-2_{D205} behaves like HIV-1, in which no dimer formation has been observed.

To emphasize both the alternative subtype of HIV-2_{D205} and its evolutionary location close to a hypothetical common ancestor of the HIV-2/SIV_{sm}/SIV_{mac} group of lentiviruses, we propose that the virus is called HIV-2_{ALT}.

In our evolutionary tree, HIV-2_{ALT} is closely related to this common ancestor and branches earlier than SIV_{sm}/SIV_{mac} and the HIV-2 prototypes. Thus, the question of the origin of HIV-2 is open again. It is still unclear whether the host of a common ancestor of the HIV-2/SIV_{sm}/SIV_{mac} group was human or simian. A horizontal infection of a human by sooty mangabey seemed plausible because of the extent of sequence identity and the endemic coincidence^{1,15}. But it must be noted that SIV_{sm} and SIV_{mac} are viruses taken from captive animals and that sequence data of SIV_{sm} from wild animals have not yet been published. Because captive monkeys were injected with human material back in the 1960's, artificial transmission from a human to a simian host could have occurred. It is possible, therefore, that SIV_{sm} and SIV_{mac} are fundamentally human viruses. In addition, the finding of HIV-2_{ALT}, a virus in a human, which

is evolutionarily older than SIV_{sm}, could indicate that all subtypes of the HIV-2/SIV_{sm}/SIV_{mac} group are of human origin.

On the basis of epidemiological data it has been proposed¹⁶ that HIV-2, or at least a subtype of HIV-2, is less pathogenic in humans than is HIV-1. HIV-2_{ALT}, originating from a healthy Ghanaian, could be of this kind, as its position in the tree indicates that there exist viruses that had time to adapt to their human hosts. In addition, some individuals could have already evolved protective mechanisms that prevent certain HIVs from causing lethal disease. But out of the fifty cases of HIV-2 infection diagnosed in Germany so far, three have been fatal, one of them showing exclusively neurological symptoms¹⁷. Clearly, among viruses that serologically react like HIV-2, there are strains with very different genetic and biochemical properties. Thus, for further investigations on the spread of HIV-2 and its pathogenicity, it may be important to distinguish between the prototypic HIV-2 strains and the HIV-2_{ALT} subtypes. □

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Isolation and characterization of the G-actin-myosin head complex

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THE two main proteins involved in muscular contraction and cell motility, myosin and actin, possess the intrinsic property of being able to form filamentous structures¹. This property poses a serious impediment to the study of their structures and interactions, and a considerable effort has thus been made to isolate their functional domains. The globular part of myosin, subfragment-1 (S1), which possesses ATPase and actin-binding sites as well as supporting the movement of actin filaments during *in vitro* assays, has been isolated^{2–4}. But because S1 is efficient in inducing actin polymerization^{5,6}, as is myosin, it has not been possible to prepare and characterize a complex of S1 with monomeric actin (G-actin). We have now used chromatographically purified proteins to show that only the S1 isoenzyme carrying the A1 light-chain subunit promotes actin polymerization. The other isoenzyme, S1 (A2), carrying the A2 light-chain subunit, binds to actin, forming a tight complex of G-actin and S1 in a 1:1 ratio. This new functional difference

TABLE 2 Sequence conservation of genetic units (nucleotide/amino-acid)

HIV-2 _{D205} genetic unit	HIV-2 _{ROD}	% Identity to:	
		SIV _{sm}	SIV _{mac251}
U3	72.9	66.8	63.5
R	95.4	94.3	91.6
U5	88.9	87.2	87.1
<i>gag</i>	81.5/84.5	82.5/87.2	80.6/83.7
<i>pol</i>	79.0/82.0	84.7/82.4	77.4/77.1
<i>vif</i>	72.4/68.9	72.9/68.7	72.0/61.0
<i>vpx</i>	76.1/75.2	76.4/75.2	75.2/77.9
<i>vpr</i>	78.8/69.8	74.6/73.4	78.9/76.4
<i>tat</i> exon 1	78.4/66.3	76.0/60.5	81.1/66.3
<i>rev</i> exon 1	76.1/56.5	64.1/52.4	70.0/60.9
<i>env</i> (partial)	70.0/67.0	68.8/65.6	68.6/65.7
<i>nef</i> (partial)	73.5/72.1	66.5/62.8	66.6/58.3

Identity between HIV-2_{D205,7} and HIV-2_{ROD}, SIV_{sm} and SIV_{mac} allocated to single genes and LTR regions; *nef* from SIV_{sm} extends further downstream and was only compared up to the position corresponding to the stop codon of HIV-2 and SIV_{mac}.

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between myosin isoforms directly implicates the A1 light-chain in myosin-induced actin polymerization. Additionally, this finding should lead to the purification of the stable G-actin-S1 complex needed to resolve the structure and to understand the molecular dynamics of the actin-myosin system.

Two principal myosin isoforms have been defined on the basis of the heterogeneity of the 'alkali' light-chain subunits, A1 and A2, bound to the globular heads of myosin⁷. Thus the two isoforms of the skeletal myosin head, S1(A1) and S1(A2), differ in the composition of their light chains: the A1 subunit of S1(A1) has an additional 41 N-terminal amino acids⁸. At low ionic strength, this difference alters both the actin-activated Mg^{2+} -dependent ATPase of S1, and the affinity of S1 for filamentous actin (F-actin)⁹⁻¹¹. In more physiological conditions, and in the presence of the muscle regulatory system, this difference results mainly in a higher binding of S1(A1) isoenzyme to F-actin¹².

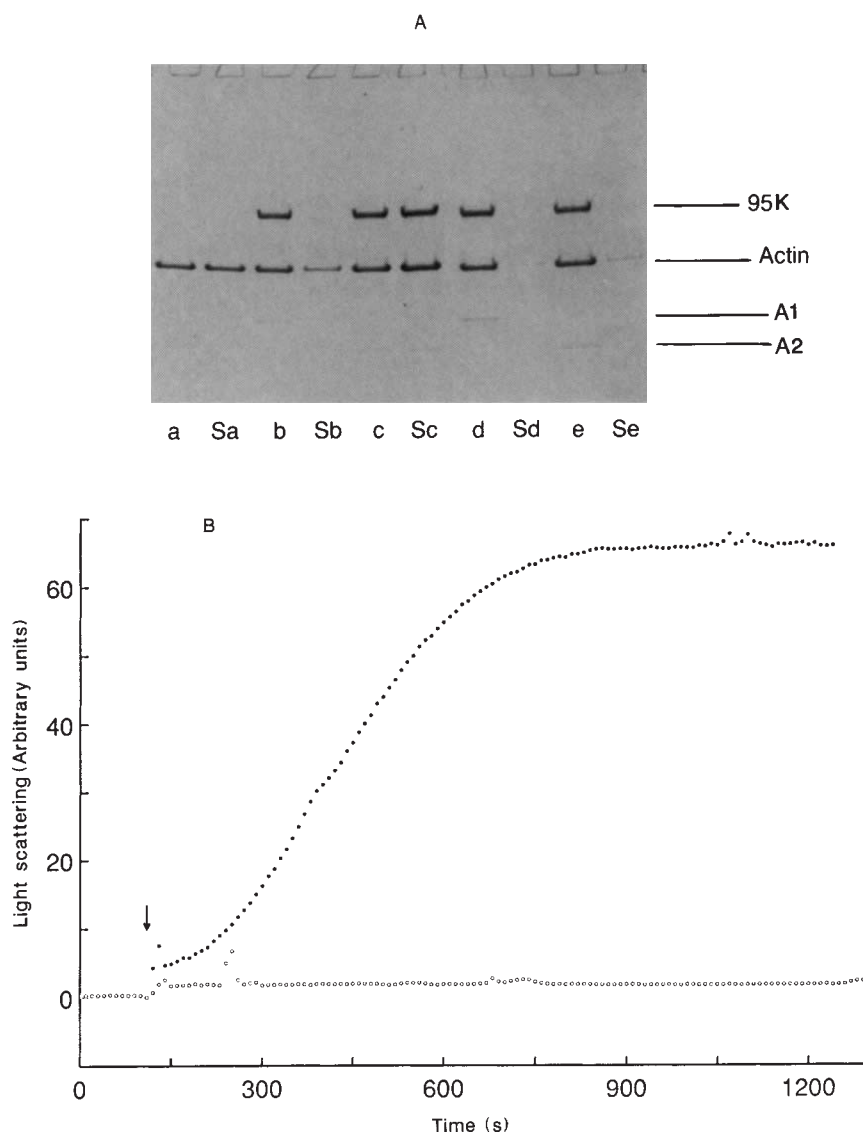
We first investigated whether both isoenzymes of S1 polymerize actin in the same fashion by measuring the amount of protein polymerized by each isoenzyme after a high-speed centrifugation (Fig. 1A). The addition of S1(A1) to a solution of G-actin induced the formation of filamentous actin co-sedimenting with an equimolar amount of S1(A1). When we used S1(A2), however, both actin and S1(A2) remained in the supernatant, indicating that actin polymerization had not occurred. By contrast, control experiments showed that both S1(A1) and S1(A2)

normally bind to F-actin and cosediment with it (Fig. 1A, lanes d, Sd, e, Se). We established unequivocally the lack of actin polymerization in the presence of S1(A2) by performing light-scattering experiments (Fig. 1B). The addition of S1(A2) to G-actin did not modify the intensity of scattered light, confirming the absence of actin polymer in the solution. By contrast, S1(A1) gradually increased the intensity of light-scattering of the actin solution, demonstrating that actin polymerization had occurred. Further analysis of the last result showed that, in contrast to a previous report¹³, the time course of the S1(A1)-induced polymerization resembles that of salt-induced polymerization¹⁴—that is, a lag phase, usually interpreted as the activation and nucleation phase of the actin monomer, precedes the elongation phase of the actin polymer.

Although S1(A2) does not polymerize actin, it does form a tight complex with monomeric actin. By measuring fluorescence polarization of chromophoric labels attached to actin, we could detect changes in the hydrodynamic volume of the protein, normally occurring during the formation of G-actin-S1 complexes or during actin polymerization¹⁵. When S1(A2) was added to G-actin labelled with 1,5-IAEDANS(5-[2-(iodoacetyl)-aminoethyl]aminonaphthalene-1-sulphonic acid), the polarization rapidly increased from its initial value of 0.23 to 0.33 (Fig. 2a). These values agree reasonably well with theoretical calculations, which give polarization values of 0.22 and 0.345 for the

FIG. 1 A, G-actin polymerization followed by co-sedimentation experiments. a, G-actin alone (18 μ M); b, d, G-actin mixed with S1(A1) (15 μ M); or c, e, S1(A2) (15 μ M); were centrifuged for 60 min at 160,000g after incubation for 30 min at 20 °C in G buffer (a, b, c) or in G buffer supplemented with 2 mM $MgCl_2$, 0.2 mM EGTA (d, e). Aliquots were withdrawn before (a-e) and after (Sa-Se) centrifugation and were loaded on 3.5-18% gradient polyacrylamide gel. Position of S1 heavy chain shown as 95K (M_r 95,000). B, G-actin polymerization detected by light scattering. Time course of light scattering at 90° ($\lambda_{excitation}$ = 600 nm) of G-actin (11 μ M) in G buffer observed after the addition (arrow), of 13 μ M S1(A1) (●) or S1(A2) (○).

METHODS. S1 isoenzymes S1(A1) and S1(A2) were prepared from rabbit skeletal muscle myosin as previously reported¹⁹ and were kept on ice in Tris-HCl buffer (30 mM), NaH_2PO_4 (0.1 mM), dithioerythritol (0.2 mM), pH 7.8. G-actin was extracted from the acetone powder of muscle of the same type as S1 (ref. 20), and further purified by G-150 gel chromatography using a column equilibrated with G buffer (Tris-HCl buffer, 2 mM; $CaCl_2$, 0.2 mM; ATP, 0.1 mM; DTE, 0.2 mM; NaH_2PO_4 , 0.1 mM, pH 7.8). Protein concentrations were determined from absorbance^{10,21}.



monomeric actin and the G-actin-S1 complex, respectively¹⁶. For S1(A1), the polarization increased initially to 0.37, but rather than remaining stable, it further slowly increased to 0.44, the value characteristic for F-actin. We interpreted these observations as follows. When either of the S1 isoenzymes is mixed with G-actin, it forms a G-actin-S1 complex. Whereas the G-actin-S1(A2) complex does not change its aggregation state, the G-actin-S1(A1) complex has a strong tendency to form filamentous structures. This ability of the complex to polymerize must be due to the presence of the extra N-terminal residues of the A1 light chain, which were shown to directly interact with amino-acid residues 360, 362 and 364 of actin^{17,18}. Whether actin polymerization results in the binding of the A1 light chain and the heavy chain to the same or to different actin monomers remains to be determined. Fluorescence polarization data, however, support the formation of a 1:1 G-actin-S1 complex in an early stage of the polymerization process. Furthermore, from fluorescence anisotropy titration of G-actin with S1(A2) (Fig. 2b), we determined that the number of S1(A2)-binding sites on G-actin per G-actin monomer is 1.1 and that the dissociation constant (K_d) for the complex formation is $0.4 \mu\text{M}$.

Because of the sensitivity of the G-actin-S1(A2) complex to ATP, it is not possible to purify the complex in the ATP-containing buffer needed for G-actin stability. Therefore we developed a method of preparing a covalent G-actin-S1(A2) complex by crosslinking G-actin to S1(A2) at 4°C using EDC (1-ethyl-3[3-(dimethylamino)-propyl]carbodiimide) as a catalyst. The purification of this complex by gel filtration chromatography yielded a product that was contaminated by traces of S1(A2) but entirely free of monomeric actin (Fig. 3). This complex was eluted from the column in about the same volume as was the relative molecular mass (M_r) standard (M_r 158,000), and therefore was certainly composed of G-actin and S1 in a 1:1 ratio (M_r 155,000), corroborating the results of the spectroscopic measurements.

Although a more complete investigation is needed to understand in detail the mechanism of the S1(A1)-induced actin polymerization, it is interesting to note that the A1 light chain is present only in myosin from skeletal or cardiac muscle. So, smooth muscle or nonmuscle myosins which possess A2-like light chains, may not polymerize actin. Whether this functional difference between the two S1 isoforms has physiological sig-

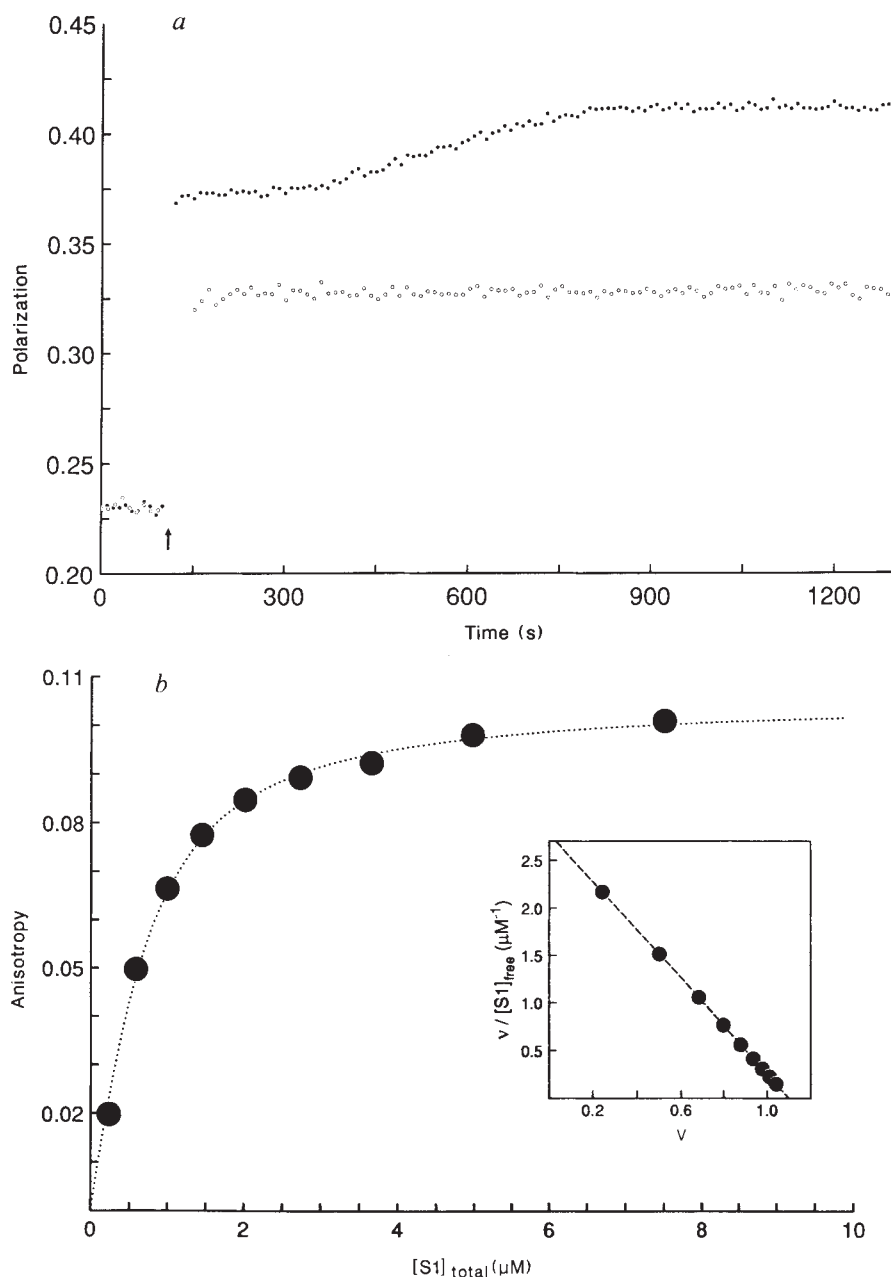


FIG. 2 a, Effect of S1(A1) and of S1(A2) on the fluorescence polarization of 1,5-IAEDANS-labelled actin. S1(A1) ($13 \mu\text{M}$; ●) or S1(A2) ($13 \mu\text{M}$; ○) were added (arrow) to a solution of 1,5-IAEDANS-labelled G-actin ($11 \mu\text{M}$) in G buffer. b, Titration curve of $0.5 \mu\text{M}$ 1,5-IAEDANS-labelled G-actin by S1(A2). The values of fluorescence anisotropy and the fraction of bound protein were calculated from polarization data as described²². Insert, Scatchard plot of the data where v is the fraction of S1 bound to actin.

METHODS. Actin was labelled with 1,5-IAEDANS according to ref. 23 and purified on a G-150 gel filtration column. Polarization measurements were performed at 20°C on an SLM 8000 fluorometer.

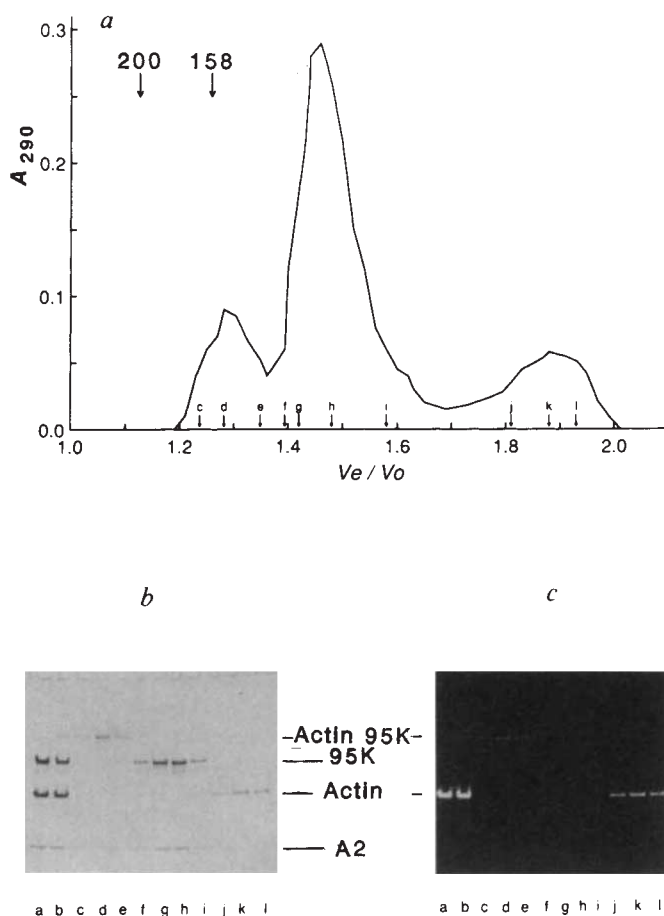


FIG. 3 Purification of the covalent G-actin-S1(A2) complex. *a*, Elution profile of EDC-crosslinked G-actin-S1(A2) complex from a Sephadex G-150 column. Abbreviations: V_0 , elution volume; V_e , void volume. Aliquots of fractions c-l were loaded on SDS-PAGE and stained with Coomassie blue (*b*) or viewed in the ultraviolet light (*c*). Lanes a and b, composition of 1,5-IAEDANS-labelled G-actin-S1(A2) mixture before and after EDC-catalysed crosslinking reaction, respectively.

METHODS. 1,5-IAEDANS-labelled G-actin (40 μ M) and S1(A2) (50 μ M) were mixed in G buffer at 4 °C and a solution of EDC was added to a final concentration of 10 mM. During the 50 min of the crosslinking reaction, the mixture was centrifuged at 160,000g, 4 °C. The supernatant was then collected, the reaction was stopped with a 100-fold molar excess of DTE, and the sample was loaded on a G-150 column (2.5 $\times$ 140 cm) equilibrated with G buffer at 4 °C. M_r standards ($M_r \times 10^{-3}$): aldolase, 158; α -amylase, 200.

nificance is not clear, because in the presence of 0.5 mM $MgCl_2$, the G-actin-S1(A2) complexes spontaneously form filaments (data not shown). Nonetheless, both the noncovalent and covalent complexes described here are undoubtedly useful for further functional and structural studies, as they provide potential samples for the crystallization and the resolution of the three-dimensional structure of the native G-actin-myosin head complex. □

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Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*

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CYCLOSPORIN A, a cyclic fungal undecapeptide produced by *Tolypocladium inflatum*, is a potent immunosuppressive drug originally isolated as an antifungal antibiotic^{1,2}. Cyclosporin A (CsA) is widely used in humans to prevent rejection of transplanted organs such as kidney, heart, bone marrow and liver³. The biochemical basis of CsA action is not known: its primary cellular target has been suggested to be calmodulin^{4,5}, the prolactin receptor^{6,7} or cyclophilin, a CsA-binding protein originally isolated from the cytosol of bovine thymocytes⁸⁻¹⁰. Cyclophilin has been shown to be a highly conserved protein present in all eukaryotic cells tested¹¹⁻¹⁵ and to be identical^{16,17} to peptidyl-prolyl *cis-trans* isomerase^{18,19}, a novel type of enzyme²⁰ that accelerates the slow refolding phase of certain proteins *in vitro*^{21,22}. We demonstrate that in the lower eukaryotes *N. crassa* and *S. cerevisiae*, cyclophilin mediates the cytotoxic CsA effect. In CsA-resistant mutants of both organisms, the cyclophilin protein is either lost completely or, if present, has lost its ability to bind CsA.

We selected for CsA-resistant mutants in the St Lawrence 74A wild-type strain of *N. crassa* by applying the lowest CsA concentration (1 μ g ml⁻¹) found to prevent growth. Forty-one ultraviolet-induced mutant strains were isolated and four of those, belonging to a high-resistance class (B9; B12; B27; B32; all growing on 10 μ g ml⁻¹ CsA), were crossed to a double-mutant strain *pan-2 arg-12* to combine the CsA-resistance with 'forcing markers' for heterokaryon formation. We performed the same procedure with a spontaneous mutant (B60) which grew on 5 μ g ml⁻¹ CsA. In all five crosses, CsA-resistant and CsA-sensitive segregants occurred in a 1:1 ratio, indicating the involvement of a single gene in each case. Segregants that carried each mutation in wild-type background were selected for biochemical analysis (Fig. 1); segregants with mutations in combination with either *pan-2* or *arg-12* markers and mating type *A* were selected for dominance and complementation tests. We found all five CsA-resistant mutations to be recessive in mycelia that were heterokaryotic for either one of them and the CsA-sensitive wild-type allele. Mycelia heterokaryotic for pairs of CsA-resistant mutations displayed the CsA-resistant phenotype, that is, they were unable to complement each other. Apparently the five mutations are allelic, indicating that a single gene, which we name *csr-1*, is responsible for CsA-resistance. Because we noticed a weak linkage (~33%) between CsA-resistant mutations

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and mating type *a/A* alleles (located on *N. crassa* linkage group I), mutant B12 was crossed to markers on chromosome I, that is *nit-2* (left arm) and *his-2* (right arm). We observed linkage with *his-2* with 19.5% recombination. The linkage with the mating type locus, and *his-2* allows us to position *csr-1* distal from *his-2* on the right arm of chromosome I.

All mutants grew normally at 25 °C. We also tested them for growth at 29 °C and 37 °C, but no temperature-sensitivity was observed.

In wild-type strains of *N. crassa* cyclophilin is located in both cytosol and mitochondria¹⁴. Both forms are encoded by a single nuclear gene, which is transcribed into two messenger RNAs with different 5'-ends. The shorter mRNA codes for the cytosolic form of relative molecular mass 20,000 (*M*, 20K), whereas the longer mRNA codes for a 24K precursor protein whose N-terminal presequence is cleaved, in two steps, on import into mitochondria, leading to the mature 20K cyclophilin protein in the matrix¹⁴.

To analyse whether the mutations leading to CsA-resistance affected the levels of cyclophilin, we performed western blot assays²³ with cyclophilin-specific antibodies¹⁴, as well as CsA-binding assays^{8,9,11} (Fig. 1). Both cytosolic and mitochondrial fractions of wild-type strain and the CsA-resistant segregants in wild-type background (to ensure that only the *csr-1* gene was affected), were analysed. Cyclophilin was present in both the cytosolic and mitochondrial fractions of the wild-type strain 74A and the spontaneous mutant B60. None of the other mutants, however, contained immunodetectable cyclophilin in either the cytosol or the mitochondria (Fig. 1a).

In all the mutants we tested (including B60), no significant CsA-binding activity was detectable in the cytosolic fractions (Fig. 1b), in contrast to wild-type cytosol where normal binding was observed¹⁴. In mitochondrial extracts of all mutant strains (prepared by lysing isolated mitochondria in 1.8% *N*-octylglucoside and centrifugation¹⁴), specific CsA-binding activity was

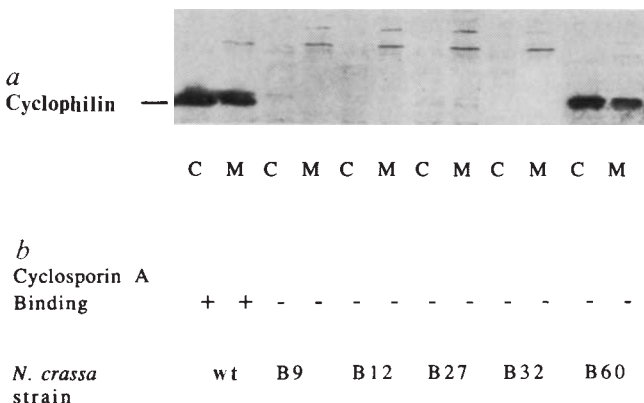


FIG. 1 Western blot analysis of cyclophilin (20K) (a) and analysis of CsA-binding (b) of cytosolic and mitochondrial fractions of *N. crassa* wild-type and CsA-resistant mutants.

METHODS. a, *N. crassa* 74A wild-type (wt) and CsA-resistant mutants (B9, B12, B27, B32, B60) were fractionated into cytosol (C) and mitochondria (M) (refs 14, 26). Protein (100 µg) from each fraction was electrophoresed on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose paper²³, and immunodecorated with polyclonal antibodies raised against *N. crassa* cyclophilin¹⁴. Bound antibodies were visualized using peroxidase coupled to antibodies against rabbit IgG. b, Cytosolic fractions of the different strains were assayed for CsA binding^{8,9,11,14}. With all mutants tested, specific binding of [³H]CsA was less than 5–10% of that of wild-type cytosol¹⁴. Isolated mitochondria were lysed in 1.8% *N*-octylglucoside, centrifuged¹⁴ and the detergent extracts tested for the ability to bind [³H]CsA in a LH-20 assay^{8,9,11,14}. In the mitochondrial extracts of the different mutants, specific binding of [³H]CsA was significantly reduced (10–30% of specific binding of wild-type mitochondrial extracts¹⁴).

reduced to 10–30% of that seen with wild-type mitochondrial extracts¹⁴. The residual binding activity present in the mutant mitochondria may be due to another CsA-binding component, different from cyclophilin.

Taken together, CsA-resistant mutants of *N. crassa* that are affected in the gene *csr-1* (which is probably the gene coding for cyclophilin) had either completely lost cyclophilin in both cytosol and mitochondria, or, if the protein was still present (as in mutant B60), it had lost its CsA-binding ability. These data indicate that either cytosolic or mitochondrial cyclophilin, or both, is the mediator of CsA action in *N. crassa*.

We performed similar experiments in the yeast *S. cerevisiae*, which was previously reported to be insensitive to CsA (ref. 1). We found that CsA-sensitivity seems to be coupled to a Petite phenotype, and is most probably due to alterations in the cell wall²⁴ which lead to increased permeability to the hydrophobic drug. Forty-eight different laboratory strains of *S. cerevisiae* were tested for CsA-sensitivity; only two, both with the Petite phenotype, were found to be sensitive to 100 µg ml⁻¹ CsA. CsA-sensitive mutants could easily be selected from CsA-resistant wild-type strains by screening for spontaneous Petite phenotypes.

Starting with the CsA-sensitive ρ^0 strain IL 993/5c (ref. 25) (wild type (wt); Fig. 2), eight independent, spontaneous CsA-resistant mutants were isolated (strains 1–8; Fig. 2). Growth tests on nonfermentable carbon sources such as glycerol, confirmed that they did not represent ρ^+ revertants. All CsA-resistant yeast mutants showed no significant CsA-binding activity in the cytosolic fractions (Fig. 2b). In addition, two of these mutants (mutants 7 and 8; Fig. 2a) contained no immunodetectable cyclophilin. Yeast cyclophilin has an apparent relative molecular mass of 17K, and is recognized by antibodies raised against either *N. crassa* cyclophilin¹⁴ or isolated yeast cyclophilin. We have cloned and sequenced both a full-length complementary DNA and the gene coding for the cytosolic form of *S. cerevisiae* cyclophilin (K. Dietmeier and M. T., manuscript in preparation).

Our results demonstrate that cyclophilin is a cellular target of CsA. Both in *N. crassa* and in yeast the cytotoxic action of CsA is mediated by cyclophilin. In the absence of cyclophilin (or in the presence of a mutated cyclophilin that has lost its CsA-binding activity), CsA does not have any toxic effect.

These findings raise several intriguing questions concerning both the physiological role of cyclophilin and the mechanism

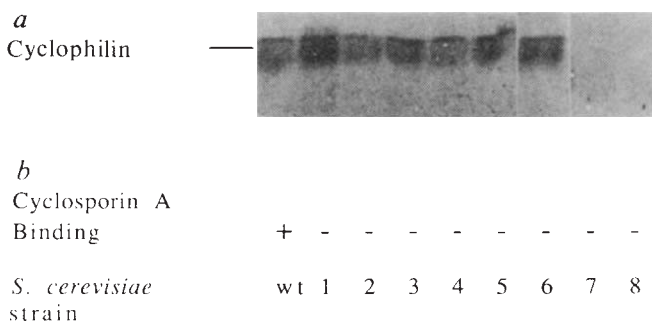


FIG. 2 Western blot analysis of cyclophilin (17K) (a) and analysis of CsA-binding (b) of cytosolic fractions of *S. cerevisiae* wild-type and spontaneous CsA-resistant mutants.

METHODS. a, Wild-type (wt) strain IL 993/5c (ref. 24) was grown at 30 °C in YPD medium³², 1% ethanol, 0.1% Tween 80; eight independent spontaneous mutants (strains 1–8) were grown under identical conditions, except that the medium contained 50 µg ml⁻¹ CsA. Zymolyase-spheroplasts were lysed and cytosolic fractions prepared³² and analysed as in Fig. 1. b, Cytosolic fractions of the different strains were assayed for CsA-binding^{8,9,11,14}. With all mutants tested (strains 1–8), specific binding of [³H]CsA was less than 5–10% of that of wild-type cytosol¹⁴.

of CsA action. Cyclophilin is a highly conserved protein in all eukaryotes analysed so far^{9,12-14}. How can *N. crassa* and yeast cells survive without showing any detectable phenotype in the absence of cyclophilin?

Recent work^{16,17} has demonstrated the identity of cyclophilin to peptidyl-prolyl *cis-trans* isomerase (PPIase)^{18,19}. Both isolated *N. crassa* and yeast wild-type cyclophilins were found to possess PPIase-activity similar to the human and bovine enzymes (S. Mayer, F. X. Schmid, D. W. Nicholson and M.T., manuscript in preparation). Furthermore, PPIase-activity measured in cytosolic and mitochondrial fractions of *N. crassa* mutants that lack cyclophilin, indicate the presence of additional PPIase-activities in both the cytosol and mitochondria. In a Southern blot analysis of *N. crassa* DNA, using low-stringency conditions, we have found two bands that cross-hybridize with a cyclophilin probe, and which may correspond to the additional PPIase-activities. The presence of these additional PPIase-activities could explain why a complete loss of cyclophilin does not lead to cell death.

Why does CsA stop growth of wild-type cells but not of cyclophilin-deficient mutant cells? We propose that binding of CsA to cyclophilin leads to the formation of a complex which then interacts with another, unidentified cellular component. This interaction must be lethal to the cell. Other explanations are possible, although less likely. For example, cyclosporin A could be modified by cyclophilin and the derivative could be lethal to the cells.

It is tempting to speculate that cyclophilin may be involved in the unfolding/refolding of newly synthesized proteins, in particular of those which have to traverse the various cellular membranes²⁶⁻²⁹. In this respect it may be relevant that a cyclophilin-related protein, namely the product of the *ninaA* gene of *Drosophila melanogaster*, has been suggested to be involved in the folding of rhodopsin *in vivo*^{30,31}.

Note added in proof: We have learned that T. Chang, B. Weisblum and R. L. Metzberg (personal communication) have also identified a *N. crassa* gene for cyclosporin A resistance on linkage group I, left of *cyh-1* (1%). □

Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases

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SELF-INCOMPATIBILITY in flowering plants is often controlled by a single nuclear gene (the *S*-gene) having several alleles¹. This gene prevents fertilization by self-pollen or by pollen bearing either of the two *S*-alleles expressed in the style. Sequence analysis shows that three alleles of the *S* gene of *Nicotiana alata* encode style glycoproteins^{2,3} with regions of defined homology. Two of the homologous regions also show precise homology with ribonucleases T₂ (ref. 4) and Rh (ref. 5). We report here that glycoproteins corresponding to the *S*₁, *S*₂, *S*₃, *S*₆ and *S*₇ alleles isolated from style extracts of *N. alata*⁶ are ribonucleases. These style *S*-gene-encoded glycoproteins account for most of the ribonuclease activity recovered from style extracts. The ribonuclease specific activity of style extracts of the self-incompatible species *N. alata* is 100–1,000-fold higher than that of the related self-compatible species *N. tabacum*. These observations implicate ribonuclease activity in the mechanism of gametophytic self-incompatibility.

Cloned complementary DNAs corresponding to the *S*₂, *S*₃- and *S*₆-allelic glycoproteins⁶ have recently been isolated^{2,3}. The primary amino-acid sequences of three *S*-glycoproteins show some regions that are perfectly conserved and other regions that are less homologous³. Sequence analysis reveals significant homology between the *S*-glycoproteins and the extracellular ribonucleases (RNases) T₂ of *Aspergillus oryzae*⁴ and Rh of *Rhizopus niveus*⁵ (Fig. 1). The two longest regions of homology, residues 67–72 and 138–142 (Fig. 1) include His 67 and His 139 which are implicated in RNase T₂ catalysis⁴. It is significant that these homologous regions are also conserved among the three *S*-glycoproteins. In total, of the 122 amino acids perfectly conserved among the three *S*-glycoproteins, 30 are aligned with identical amino acids in the fungal RNases and another 22 are aligned with closely related amino acids. The positions of 5 out of 10 cysteine residues are conserved between the *S*-glycoproteins and the RNases. Two of these cysteine residues, 86 and 143, located in the vicinity of the proposed RNase active site, form a disulphide bond in RNase T₂ (ref. 4). These sequence similarities indicate a close structural relationship between the active-site domain of the two secreted fungal RNases and the homologous region of the *S*-glycoproteins. This homology prompted us to investigate the RNase activity of the *S*-glycoproteins.

The *S*-glycoproteins can be purified from style extracts by a procedure involving ammonium-sulphate fractionation and cation-exchange chromatography⁶. The *S*-glycoproteins isolated by this method behave as a single species on SDS-PAGE or reverse-phase HPLC; N-terminal sequencing confirms their identity with the proteins predicted from the cloned cDNAs⁶. The association of particular *S*-glycoproteins with the corresponding *S* allele is further supported by data which show that, in every case examined, the elution profile of proteins resolved by cation-exchange chromatography correctly predicts the genotype of the source material. Also, these isolated *S*-glycoproteins are active as inhibitors of *in vitro* pollen tube

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Because most of the RNase activity in crude style extracts was applied to the cation-exchange column (Fig. 2 legend) the percentage of the RNase activity recovered from the column was a good indicator of the proportion of the total style activity that is attributable to the *S*-glycoproteins. Of the recovered RNase activity, 40% (Fig. 2c) to 86% (Fig. 2b) can be attributed to *S*-glycoproteins. *N. alata* style extracts also contain an additional minor RNase activity (X in Fig. 2) which does not co-fractionate with the *S*-glycoproteins on chromatography and is therefore not an allele-specific RNase.

	Style extract*	Pollen extract*
<i>N. alata</i>		
S_1S_1	180	0.7
S_2S_2	240	—
S_3S_3	400	—
S_6S_6	300	0.7
S_7S_7	1,400	—
<i>N. tabacum</i>		
cv. Wisconsin 38	1.4	

* A_{260} units released per min per mg.

The nature of gametophytic self-incompatibility indicates that the *S*-gene must be expressed in the pollen as well as in the style¹. Relative to style extracts, only trace amounts of RNase activity were detected in pollen extracts (Table 1) and in pollen germinated *in vitro* (B.A.M., unpublished results) indicating that, if it is an RNase, the product of the *S* gene in pollen is not highly expressed. Indeed, it now seems very unlikely that the *S*-gene products in the *N. alata* pollen and style are identical, yet it is clear that they must share an allelic specificity for recognition to occur¹. It is possible that the specificity controls the access of style RNase to the putative substrate within the pollen tube. Alternatively, the specificity could reside in the

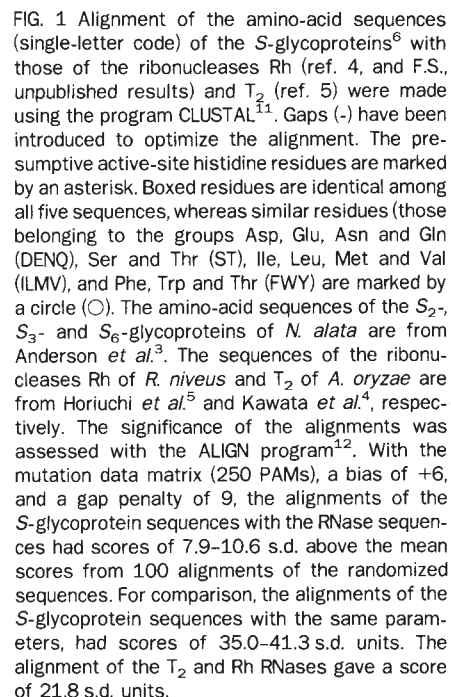
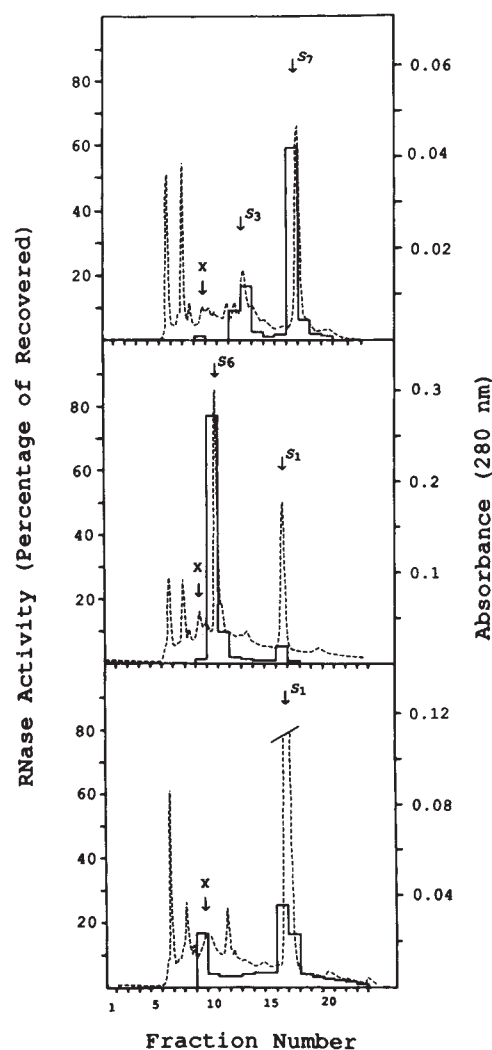


FIG. 2 Elution profiles of style extracts from mature flowers of *N. alata*. Extracts from styles of plants homozygous for the S_1 -, S_3 -, S_6 - and S_7 -alleles were prepared and fractionated by cation-exchange chromatography as previously described⁶. The elution profile was continuously monitored for absorbance at 280 nm (dotted line) and each fraction was assayed for ribonuclease activity⁸ (solid line). *a*, Fractionation of an extract of a mixture of five styles, each of flowers homozygous for the S_3 - and S_7 -alleles. *b*, Fractionation of an extract of a mixture of 10 styles each of flowers homozygous for the S_6 - and S_1 -alleles. *c*, Fractionation of an extract from 20 styles of flowers homozygous for the S_1 -allele. The elution position of each of the *S*-glycoproteins⁶ is indicated. In each case, a major peak of ribonuclease activity co-fractionated with the *S*-glycoproteins on chromatography. A second peak of ribonuclease activity, not associated with the *S*-glycoproteins, is indicated by X. The ammonium-sulphate fractionation and desalting steps seem to cause no more than a 20% loss of activity. Thus 80% of the RNase activity in crude style extracts was subjected to cation-exchange chromatography. Total recovered activities were 64, 172 and 43 units in *a*, *b* and *c*, respectively. Conditions for isolation of the glycoproteins was as published⁶ without further optimization for recovery of enzyme activity.

METHODS. Styles from defined *S*-genotypes of *N. alata*² were collected from plants maintained in the glasshouse. Extracts were prepared by homogenization (200 mg ml⁻¹) in 100 mM Tris-HCl buffer, pH 7.8, 14 mM 2-mercaptoethanol containing 20 mg ml⁻¹ Polyclar AT and 20 mg ml⁻¹ Dowex 1 × 2 Cl (ref. 6). *S*-glycoprotein recovered from the extract after ammonium-sulphate fractionation (50–95%) was desalted into 50 mM sodium acetate, pH 5, by passage over a Sephadex G-25 column (Pharmacia) and applied to a Mono-S (HR 5/5) cation-exchange (Pharmacia) column. The column was developed with a linear NaCl gradient (0–500 mM at 1 ml min⁻¹) at room temperature (~18 °C). The eluate was continuously monitored for absorbance at 280 nm (dotted line). An aliquot of each 1-ml fraction was assayed for RNase activity essentially as previously described⁸, except that the reactions were carried out in 100 mM imidazole-HCl, pH 7, 50 mM KCl at 37 °C for 30 min using torula yeast RNA (4 mg ml⁻¹; Sigma) as substrate. To reduce endogenous RNase activity, the substrate was repeatedly extracted with phenol:chloroform:isoamyl alcohol (25:24:1)¹³, subjected to LiCl precipitation¹⁴ and finally precipitated as the ammonium salt.



inhibition of the style RNase by the gametophyte.

This mechanism of inhibition of pollen tube growth is compatible with some observations on unilateral interspecific incompatibility¹. One type of unilateral incompatibility is exemplified by reciprocal crosses between the self-incompatible species *N. alata* and the self-compatible species *N. tabacum*. Styles of all *S*-genotypes of *N. alata* have high RNase activity (Table 1) and support only limited growth of pollen tubes from *N. tabacum*. By contrast, styles from *N. tabacum* allow growth of pollen tubes from all *S*-genotypes of *N. alata*, leading to seed-set. It is significant that styles from *N. tabacum* do not contain an *S*-glycoprotein-like component and have relatively low total RNase activity (Table 1). The high level of RNase associated with the style *S*-glycoproteins could contribute to the rejection of *N. tabacum* pollen by mature *N. alata* styles (that is *N. tabacum* pollen could lack the ability to reject or inhibit the *S*-glycoprotein RNase). Conversely, *N. tabacum* could accept *N. alata* pollen by virtue of the absence of style *S*-glycoprotein RNase. Preliminary findings consistent with this view are that another gametophytic self-incompatible plant within the solanaceae *Lycopersicon peruvianum* has a high level of style RNase, whereas a closely related self-compatible accession has a low level of RNase activity (I. Lewis and B.A.M.-C., unpublished results). Also, bovine pancreatic RNase A is a nonspecific inhibitor of *in vitro* pollen tube growth, indicating that pollen tubes are susceptible to extracellular RNases (M. Lush, unpublished observation).

The results described here demonstrate that the *S*-glycoproteins have inherent RNase activity and indicate that

this activity is involved in the inhibition of pollen tube growth in the self-incompatibility reaction of *N. alata*. This re-orientates our thinking about the function of the *S*-glycoproteins away from the traditional receptor-ligand model¹ to models based on cytotoxic enzyme activity. Indeed, these observations lead to a more generalized hypothesis for inhibition of pollen tube growth based on uptake by the gametophyte of a cytotoxic agent secreted by the style. In this model, the pollen *S*-gene product would enable non-self pollen to reject or inactivate the style cytotoxin, whereas pollen lacking an *S*-allele or bearing an *S*-allele identical to one present in the style would be unable to perform this function. □

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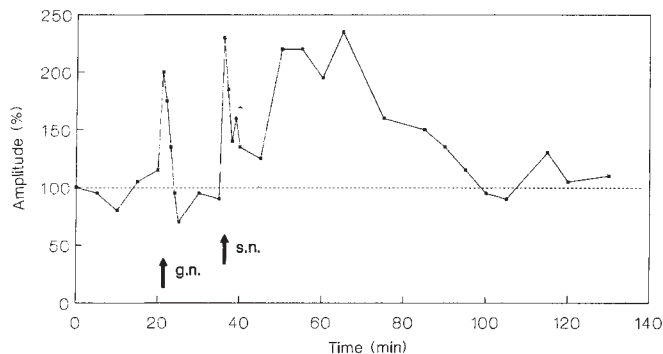
ERRATUM

Changes in spinal cord reflexes after cross-anastomosis of cutaneous and muscle nerves in the adult rat

Stephen B. McMahon & Patrick D. Wall

Nature 342, 272-274 (1989)

OWING to editorial errors, an incorrectly labelled version of Fig. 2 was used in this manuscript, with the labels for the sural nerve (s.n.) and gastrocnemius nerve (g.n.) transposed. The figure is shown in its correct form below. ☐



CORRECTIONS

An optical yield that increases with temperature in a photochemically induced enantiomeric isomerization

Yoshihisa Inoue, Taizo Yokoyama, Noritsugu Yamasaki & Akira Tai

Nature 341, 225-226 (1989)

IT has come to *Nature's* attention that this paper substantially duplicates a paper from the same authors in the *Journal of the American Chemical Society* 111, 6480-6482 (1989). *Nature* was not made aware of the latter paper by the authors although it was submitted first. ☐

The winter solstice phenomenon at Newgrange, Ireland: accident or design?

T. P. Ray

Nature 337, 343 (1989)

OWING to circumstances beyond *Nature's* control, cooperation on the above project, an account of which appeared in our issue of 26 January 1989, was not acknowledged. Professor T. P. Ray would now like to correct this omission by stating that the name of T. M. O'Brien, consultant orthopaedic surgeon in Dublin, should be added to his own. Both were simultaneously engaged on research at Newgrange. Mr. O'Brien undertook the photographic and architectural surveys, Professor Ray the astronomical calculations of the Sun's path into the chamber at the solstitial winter sunrise. ☐

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